

Examining an Animal Model of Depression with Recurrent Depression-like Episodes

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By

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ABSTRACT

Depression is a complex psychiatric disorder. It is recognized as a cyclical disease with multiple episode relapses. Many preclinical animal models have been utilized to study the behavioral and neurobiological characteristics of depression, however, almost without exception, these models do not recapitulate the recurrent nature of this disorder. It is suggested that the relationship between major depression and stress, which is the important risk factor for depression, changes over time: the depressive episodes either become increasingly autonomous of stress and occur spontaneously, or the depressed individual becomes increasingly sensitized to stress so that smaller stressors can trigger subsequent episode relapses. Therefore, a close examination of potential mechanisms evoking recurrent depressive episodes is needed.

The primary goals of this dissertation were to develop an animal model of recurrent depression using repeated and cyclic corticosterone (CORT) administration and to gain a better understanding of the consequences of such treatment on behavioral and neurobiological alterations in rodents. To begin, in Chapter 2, I examined the effect of two cycles of repeated CORT injections on rat behavior measured via repeated forced swim testing. I found that CORT produced increasingly greater effects on the depression-like behavior of rats during the second cycle of CORT exposure. These data suggested the development of a sensitized behavioral vulnerability in rats following CORT re-exposure. In Chapter 3, non-repeated behavioral testing of naïve rats was employed to examine the influence of cyclic CORT treatment on several measures of depression-like behavior, the number and maturation rate of immature dentate granule cells, as well as changes in hippocampal REST protein expression. Consistent with the results of the first experiment, CORT produced larger increases in depression-like and anhedonia-like behaviors in second

and third cycles, without changes in general locomotor behavior. Aggravation of depression-like behavior was accompanied by an accumulated decline in neurogenesis and dendritic complexity of newborn neurons in the dentate gyrus. Additionally, I discovered that glucocorticoid treatment also exerted its adverse effects on transcriptional regulation in the hippocampus, characterized by hippocampal REST protein levels. Finally, Chapter 4 included a preliminary examination of the effects of cyclic CORT exposure on changes in hippocampal microglia. CORT produced cumulative decreases in hippocampal microglia area fraction and cell morphology, which are closely coupled with microglial activation and neuroinflammatory processes. These changes were strongly correlated with decreases in dentate granule neuron maturation and increases in depression-like behavior, suggesting that the activation of hippocampal microglia may be associated with exacerbation of depressive symptoms and paralleled decreases in the number and complexity of hippocampal immature neuron population.

The overall findings of this dissertation provide evidence that repeated and cyclic glucocorticoid treatment can be used as an animal model of recurrent depression, as it provides a means to investigate the neurobiological changes governing depressive symptom relapse. Data presented in this dissertation further advance our understanding of the effects of CORT on brain and behavior, and provide support for a relationship between increasingly severe depression-like symptoms, decreases in neurogenesis and newborn cell maturation rate, as well as alterations in hippocampal REST levels and microglial cell activation.

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DEDICATION

This dissertation is dedicated to my husband, Andrey Kharitonov, and to my parents, Lidiya Lebedeva and Aleksey Lebedev.

Andrey, you have been a continuous source of love, support, and encouragement throughout my graduate career. You light up my life and I am deeply grateful for having you at my side.

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TABLE OF CONTENT

PERMISSION TO USE.....	i
ABSTRACT.....	ii
ACKNOWLEDGMENTS.....	iv
DEDICATION.....	v
TABLE OF CONTENT.....	vi
LIST OF FIGURES.....	xii
LIST OF TABLES.....	xiv
LIST OF ABBREVIATIONS.....	xv
 CHAPTER 1.....	 1
General Introduction.....	1
1.1. Thesis Outline.....	1
1.2. Hypothalamic-Pituitary-Adrenal Axis (HPA) and Stress Response.....	3
1.3. Depression.....	6
1.4. Recurrent Depression.....	8
1.5. Animal Models of Depression.....	11
1.6. Exogenous CORT Administration Model of Depression.....	14
1.7. Modeling Recurrent Depression in Animals.....	16
1.8. Hippocampal Anatomy and Circuitry.....	16
1.9. Adult Hippocampal Neurogenesis.....	19
1.9.1. Stages of Neurogenesis.....	20
1.10. Hippocampus, Neurogenesis and Depression.....	22
1.11. Repressor Element-1 Silencing Transcription Factor (REST) and Depression.....	24
1.12. Inflammation, Stress, and Depression.....	27
1.13. Microglia.....	30
1.14. Microglia, Stress, and Depression.....	33
1.15. Goals and objectives.....	35
 CHAPTER 2.....	 39

**Cyclical Corticosterone Administration as an Animal Model of Recurrent Depression:
Results of Repeated Forced Swim Testing..... 39**

2.1. Introduction.....	41
2.2. Materials and Methods.....	42
2.2.1. Subjects	42
2.2.2. Treatment with CORT	42
2.2.3. Body Weight	43
2.2.4. Behavioral Testing.....	43
2.2.5. Statistical Analyses	45
2.3. Results.....	46
2.3.1. Body Weight	46
2.3.2. Forced Swim Tests.....	48
2.3.2.1. Immobility.....	48
2.3.2.2. Latency to Immobility.....	50
2.3.2.3. Climbing	50
2.3.2.4. Swimming.....	51
2.3.3. FST Behaviors Controlled for Body Weight	52
2.3.3.1. Immobility to Body Weight (IBW) Ratio	53
2.3.3.2. Latency to Immobility to Body Weight (LIBW) Ratio	55
2.3.3.3. Climbing to Body Weight (CBW) Ratio	55
2.3.3.4. Swimming to Body Weight (SBW) Ratio	56
2.3.4. Changes in Recovery of Immobility Behavior Across Two Cycles of Treatment	57
2.4. Discussion	57

CHAPTER 3..... 66

**Cyclical Administration of CORT in an Animal Model of Recurrent Depression Results
in Aggravation of Depressive-like Behaviors and Accompanying Changes in
Neurobiological Markers..... 66**

3.1. Introduction.....	68
3.2. Materials and Methods.....	70
3.2.1. Subjects	70
3.2.2. Experimental Procedures	70

3.2.3. Behavioral Testing.....	71
3.2.3.1. Forced Swim Test	73
3.2.3.2. Sucrose Preference Test.....	73
3.2.3.3. Open Field Test.....	73
3.2.4. Perfusions and Tissue Preparation	74
3.2.5. Immunohistochemical Procedures and Analyses.....	74
3.2.5.1. Doublecortin (DCX)	75
3.2.5.2. REST	77
3.2.6. Statistical Analysis.....	78
3.3. Results.....	79
3.3.1. Effects of Repeated and Cyclic CORT Treatment on Body Weight	79
3.3.2. Effects of Repeated and Cyclic CORT Treatment on Forced Swim Test Behaviors	81
3.3.2.1. Immobility.....	81
3.3.2.2. Latency to immobility.....	81
3.3.2.3. Climbing	83
3.3.2.4. Swimming	83
3.3.3. Effects of Repeated and Cyclic CORT Treatment FST Behaviors Controlled for Body Weight	84
3.3.3.1. Immobility to Body Weight (IBW) Ratio	84
3.3.3.2. Latency to Immobility to Body Weight (LIBW) Ratio	86
3.3.3.3. Climbing to Body Weight (CBW) Ratio	86
3.3.3.4. Swimming to Body Weight (SBW) Ratio	87
3.3.4. Assesment of Recovery of Immobility Behavior Across Three Cycles of CORT Treatment	87
3.3.5. Effects of Repeated and Cyclic CORT Treatment on Open Field Test Behavior	88
3.3.6. Effects of Repeated and Cyclic CORT Treatment on Sucrose Preference Test Behavior	88
3.3.7. Effects of Repeated and Cyclic CORT Treatment on the total DCX+ cell number in SGZ and GCL.....	91
3.3.7.1. Effects of Repeated and Cyclic CORT Treatment on Maturation of DCX+ neurons in the Subgranular Zone and Granule Cell Layer	93

3.3.8. Effects of Repeated and Cyclic CORT Treatment on REST Levels in the Hippocampus	95
3.3.8.1 REST Expression Levels in CA Pyramidal and Granule Cell Layers	95
3.3.8.2. REST+ Cell Counts in Hippocampal Subregions	95
3.3.9. Analyses of Correlations	99
3.3.9.1. Correlations between Behavioral Measures and DCX+ Cell Counts and Maturation Rate	99
3.3.9.2. Correlations between REST (Cell Counts and Optical Density) and DCX+ Cell Counts and Maturation Rate	102
3.3.9.3. Correlations between REST (Cell Counts and Optical Density) and Behavioral Measures	103
3.4. Discussion	103
3.4.1 Repeated and Cyclic Treatment with CORT Resulted in Progressively Larger Changes in FST Behaviors	103
3.4.2. Possible Sensitization of the Effect of CORT on Sucrose Preference Across Cycles of Treatment	106
3.4.3. CORT- and Vehicle-Treated Rats did not Differ in General Locomotor Behavior	107
3.4.4. Repeated and Cyclic CORT Treatment Decreases the Number of DCX+ Cells and their Maturation Rate, in GCL and SGZ	108
3.4.5. Bi-Directional Changes in REST Immunoreactivity and the Number of REST+ Cells in Hippocampus of Rats Treated with One or Three Cycles of CORT	111

CHAPTER 4 116

Cyclical Exposure to CORT in an Animal Model of Recurrent Depression Results in Alterations of Hippocampal Microglial Cells..... 116

4.1. Introduction	118
4.2. Materials and Methods	120
4.2.1. Subjects	120
4.2.2. Experimental Procedures	120
4.2.3. Perfusions and Tissue Preparation	121
4.2.4. Immunohistochemical Procedures and Analyses	121
4.2.5. Immunohistochemical Procedures	122

4.2.6. Analyses of Immunohistochemical Staining	123
4.2.6.1. Quantitative Analysis of Microglial CD11b (OX-42) Structures in the Hippocampus	123
4.2.6.2. Morphological Analyses of Microglia in Hilus	123
4.2.7. Statistical Analyses	124
4.3. Results.....	124
4.3.1. Effect of Repeated and Cyclic CORT Treatment on Microglia Area Fraction Measures	124
4.3.2. Effects of Repeated and Cyclic CORT Treatment on Microglia Morphology	128
4.3.3. Correlation analyses.....	131
4.3.3.1. Correlations between Microglia Area Fraction and Behavioral Measures	131
4.3.3.2. Correlations between Microglia Area Fraction and DCX+ Cell Maturation Measures	131
4.3.3.3. Correlations between Microglial Cell Morphology and DCX+ Cell Maturation Measures	134
4.4. Discussion.....	135
CHAPTER 5.....	141
General Discussion.....	141
5.1. Summary of the Main Findings	141
5.2. Repeated and Cyclic Glucocorticoid Exposure as an Animal Model of Recurrent Depression.....	143
5.3. Neurogenesis and REST in Depression	145
5.4. Role of Microglia in Depression.....	148
5.5. Limitations	150
5.5.1. Can Depression Be Modeled in Laboratory Rodents?.....	150
5.5.2. Sex Differences in Depression.....	152
5.6. Future Directions	154
5.6.1. How Can the CORT Model of Recurrent Depression be Further Improved? .	154
5.6.2. Other Brain Structures that may be Involved in the Pathogenesis of Depression	155

5.6.3. How do Antidepressants and Recovery Influence Neurobiological Changes in the CORT Model of Recurrent Depression?	157
5.7. Conclusions	158
References.....	160

LIST OF FIGURES

Figure 1-1. Hypothalamic-Pituitary-Adrenal Axis.

Figure 1-2. Schematic representation of rodent hippocampal formation and its circuitry.

Figure 1-3. Dynamic morphological changes in a microglia cell.

Figure 2-1. The schematic experimental design.

Figure 2-2. The effect of one and two cycles of repeated corticosterone (CORT) injections on rat body weight.

Figure 2-3. The effect of one and two cycles of repeated corticosterone (CORT) injections on behavior in the forced swim test.

Figure 2-4. The effect of one and two cycles of repeated corticosterone (CORT) injections on the forced swim test (FST) behavior plotted by body weight.

Figure 2-5. Scatter plots of changes in immobility and immobility to body weight (IBW) ratio scores in individual rats.

Figure 3-1. The schematic experimental design.

Figure 3-2. The effect of one, two, and three cycles of repeated corticosterone (CORT) injections on body weight.

Figure 3-3. The effect of one, two, and three cycles of repeated corticosterone (CORT) exposure on behavior in the forced swim test (FST).

Figure 3-4. The effect of repeated and cyclic corticosterone (CORT) on the forced swim test (FST) behavior plotted by body weight.

Figure 3-5. The effect of one, two, and three cycles of repeated corticosterone (CORT) exposure on behavior in the open field (A) and in the sucrose preference test (B).

Figure 3-6. The effect of one, two, and three cycles of repeated corticosterone (CORT) treatment on the number of doublecortin (DCX)-positive cells in the subgranular

zone (SGZ) and granule cell layer (GCL)

Figure 3-7. The effect of one and three cycles of repeated corticosterone (CORT) treatment on the maturation of doublecortin (DCX)-positive cells in the subgranular zone (SGZ) and granule cell layer (GCL).

Figure 3-8. The effect of one and three cycles of repeated corticosterone (CORT) treatment on RE-1 Silencing Transcription factor (REST) protein density in several hippocampal regions.

Figure 3-9. The effect of one and three cycles of repeated corticosterone (CORT) treatment on the number of RE-1 Silencing Transcription factor (REST)-positive cells in several hippocampal regions.

Figure 3-10. Correlations between measures of depression-like behavior and neurobiological changes in the corticosterone (CORT) and vehicle rats in cycles one and three.

Figure 4-1. The effect of one and three cycles of repeated corticosterone (CORT) treatment on microglial area fraction measures in the granule cell layer (GCL) and hilar subregions of the dentate gyrus.

Figure 4-2. The effect of one and three cycles of repeated corticosterone (CORT) treatment on microglial area fraction measures in CA1 and CA3 hippocampal subregions.

Figure 4-3. The effect of one and three cycles of repeated corticosterone (CORT) treatment on microglial cell morphology.

Figure 4-4. Correlations between measures of microglial activation and morphology, behavior, and neurogenesis in the corticosterone (CORT) and vehicle rats in cycles one and three.

LIST OF TABLES

Table 2-1. Changes in recovery over two cycles of corticosterone (CORT) treatment.

Table 3-1. Changes in recovery over three cycles of corticosterone (CORT) treatment.

Table 3-2. Correlations between immobility in the forced swim test (FST) and RE-1 silencing transcription factor (REST) immunoreactivity in different subfields of hippocampus (cycle 1).

Table 3-3. Correlations between immobility in the forced swim test (FST) and RE-1 silencing transcription factor (REST) immunoreactivity in different subfields of hippocampus (cycle 2).

LIST OF ABBREVIATIONS

5-HT_{1A} serotonin 1A receptor

ACTH adrenocorticotrophic hormone

Arb. units arbitrary units

BDNF brain-derived neurotrophic factor

CA Cornu Ammonis

CA1.Py pyramidal cell layer of CA1

CA1.SO stratum oriens of CA1

CA1.SR stratum radiatum of CA1

CA3.Py pyramidal cell layer of CA3

CA3.SO stratum oriens of CA3

CA3.SR stratum radiatum of CA3

CA.SL stratum lacunosum of CA3

CBW climbing to body weight ratio

CD11b cluster of differentiation molecule 11B

CMS chronic mild stress

CORT corticosterone

CRH corticotropin-releasing hormone

DCX doublecortin

EC entorhinal cortex

ECT electroconvulsive therapy

FST forced swim test

GABA gamma-aminobutyric acid

GCL granule cell layer

HPA hypothalamic pituitary adrenal

Iba-1 ionized calcium-binding adapter molecule 1

IBW immobility to body weight ratio

IGF-1 insulin-like growth factor 1

IL-6 interleukin 6

LSD Fisher's least significant difference

MeCP2 methyl-CpG-binding protein 2

miRNA microRNA, micro ribonucleic acid

mPFC medial prefrontal cortex

mRNA messenger ribonucleic acid

NF-KB nuclear factor kappa B

NMDA N-methyl-D-aspartate

NPCs neural progenitor cells

OFT open field test

PFC prefrontal cortex

PVN paraventricular nucleus

REM rapid eye movement

REST repressor element-1 silencing transcription factor

SBW swimming to body weight ratio

SD standard deviation

SEM standard error of the mean

SGZ subgranular zone

SPT sucrose preference test

TBS tris buffered saline

TGF-b transforming growth factor-beta

TNF-alpha tumour necrosis factor-alpha

CHAPTER 1

General Introduction

1.1. Thesis Outline

Depression is estimated to affect approximately 350 million people worldwide (World Health Organization, 2012). In Canada, the lifetime prevalence rate of depression is approximately 11% (Patten et al., 2009). Depressive disorders often develop at a young age, typically recur and impair an individual's functioning and quality of life. For these reasons, depressive disorders are considered the leading cause of global disability in terms of years lost due to disability. Two major characteristic symptoms of depression include depressed mood and anhedonia (Nestler, Barrot, et al., 2002). Additionally, depressed individuals have increased risks of other physical illnesses, impaired social functioning, and higher morbidity (Nemeroff, 1998). Depression is further complicated by several comorbidities, such as anxiety disorders (Hirschfeld, 2001; Kessler et al., 1996; Oquendo et al., 2003; Zimmerman et al., 2013), which can influence the course of disease, delay recovery, and increase risks of relapses (Hirschfeld, 2001). Given the prevalence, and enormous personal and societal burden of depression, it is critical that research focuses on enhancing our understanding of the etiology and adequate treatment of this disorder.

The existing knowledge argues against the singular cause of depression, therefore, it is hypothesized that a combination of environmental factors (chronic stress, child adversity), psychosocial factors (personality traits, absence of social support), and biological factors (alterations in neurotransmission, hormone levels, genetics) impact the development of depression (Barnhofer & Chittka, 2010; George, Blazer, Hughes, & Fowler, 1989; Kalia, 2005; Nestler, Barrot, et al., 2002). Current antidepressant medications still rely on the

concepts of the earliest monoamine theory of depression that suggests altered activity of the monoamine neurotransmitters in depressed patients (Schildkraut, 1965). This hypothesis has faced considerable criticism over the past few decades (Ruhé, Mason, & Schene, 2007), and alternative hypotheses about the etiology of depression have been proposed. These hypotheses focus on changes in transcriptional regulation, proteins and growth factors that modulate plasticity in the adult brain. A wealth of clinical and preclinical evidence support new theories by demonstrating volumetric reductions in human brain regions known to regulate emotion and cognition (Sheline, Gado, & Kraemer, 2003), as well as decreases in cell proliferation, survival and neuronal morphology in the analogous brain regions in animal models of depression (Bambico & Belzung, 2013; Sterner & Kalynchuk, 2010). Although our knowledge of the many cellular and molecular changes associated with depression has significantly improved over several decades (Conrad, LeDoux, Magariños, & McEwen, 1999; McEwen & Magarinos, 2001), our understanding of the neurobiological mechanisms underlying the development of major depression and its continual episode relapse is still very limited.

This dissertation comprises a collection of experiments that investigates the effects of repeated and cyclic exposure to glucocorticoids on depressive behaviors and hippocampal function (brain region known to be involved in stress response). The first experiment focused on introducing an animal model of recurrent depression with repeated forced swim behavioral testing. In the second experiment, I refined the model and further examined the effects of repeated and cyclic corticosterone treatment on several measures of depressive behavior. I have also investigated the effects of glucocorticoids on neurogenesis and newborn neuron morphology in relation to treatment duration (one, two, and three cycles of 21-day corticosterone exposure). In addition, I examined alterations in REST expression in hippocampus. Finally, in the third experiment, I have assessed changes in microglial

activation in hippocampus after one and three cycles of glucocorticoid exposure to study a potential mechanism for cumulative loss of neurogenic function and sensitized depressive symptomatology in this model of recurrent depression.

The remaining sections of this chapter will provide an overview of the hypothalamic-pituitary-adrenal (HPA) axis and its relationship to major depression. Further, I will discuss how animal models provide an essential means for studying the etiology and progression of depression pathology. I will then discuss the structural and functional abnormalities linked to the depressive disorder, and the influence of stress on hippocampal plasticity and neurogenesis in relation to depression. Finally, I will give an overview of current literature on the role of hippocampal REST and microglial activation in neurogenesis and depression. This chapter concludes with a discussion of important questions that remain unanswered in the field of stress and depression research.

Chapters 2, 3 and 4 describe the experimental data that address the questions outlined in this chapter. Chapter 5 includes an overview and discussion of my experimental research, its implications and limitations, as well as directions for future investigation.

1.2. Hypothalamic-Pituitary-Adrenal Axis (HPA) and Stress Response

Stress is an significant risk factor for developing depressive disorders as described in both the clinical (Kendler, Karkowski, & Prescott, 1999; Shrout et al., 1989) and animal literature (Checkley, 1996; Cheeta, Ruigt, van Proosdij, & Willner, 1997; Daquila, Brain, & Willner, 1994; Iñiguez et al., 2014). There is increasing evidence of a strong correlation between the number of major stressful life events and the onset of depressive symptoms (Kendler et al., 1999; McLaughlin, Conron, Koenen, & Gilman, 2010; Muscatell, Slavich, Monroe, & Gotlib, 2009; Ormel, Oldehinkel, & Brilman, 2001; Parker, Schatzberg, & Lyons, 2003). Moreover, existing data demonstrate that over 80% of patients experience stress

before the onset of their depressive episode (Parker et al., 2003).

Physical and psychological stressors initiate activation of the HPA axis (Herman & Cullinan, 1997). The normal physiological stress response is well characterized in the literature. Briefly, the stress response initiates a cascade of secretory events (see Figure 1-1). Neurons in the paraventricular nucleus (PVN) of the hypothalamus release corticotropin-releasing hormone (CRH) onto the anterior pituitary, where CRH stimulates the secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary into the general bloodstream. ACTH targets the adrenal cortex, where it stimulates the production of glucocorticoids (cortisol in humans; corticosterone in rats). The released glucocorticoids help activate body functions and mobilize energy to cope with stressors. In the brain, glucocorticoids bind to mineralocorticoid (type I) and glucocorticoid (type II) receptors and exert an inhibitory effect on the PVN to terminate HPA axis activity through a negative feedback system (Herman & Cullinan, 1997).

Glucocorticoids have a widespread control over a number of body systems, such as musculoskeletal, cardiovascular, and immune systems. In addition, they also have important effects on the nervous system. The hippocampus, amygdala, and prefrontal cortex (PFC) are particularly abundant in glucocorticoid receptor expression (Feldman & Weidenfeld, 1999; Morimoto, Morita, Ozawa, Yokoyama et al., 1996). Therefore, these structures are known to be greatly involved in regulating HPA axis activity (Herman & Cullinan, 1997; Jacobson & Sapolsky, 1991; Sapolsky, Krey, & McEwen, 1984; Sapolsky, Krey, & McEwen, 1986). In particular, there is evidence to suggest that under normal physiological conditions, the hippocampus negatively affects HPA activity; in contrast, the amygdala exerts a stimulatory influence (Jacobson & Sapolsky, 1991; Nestler, Barrot, et al., 2002). For example, stimulation of the hippocampus has been shown to decrease glucocorticoid secretion, whereas stimulation of the amygdala elevated glucocorticoid levels

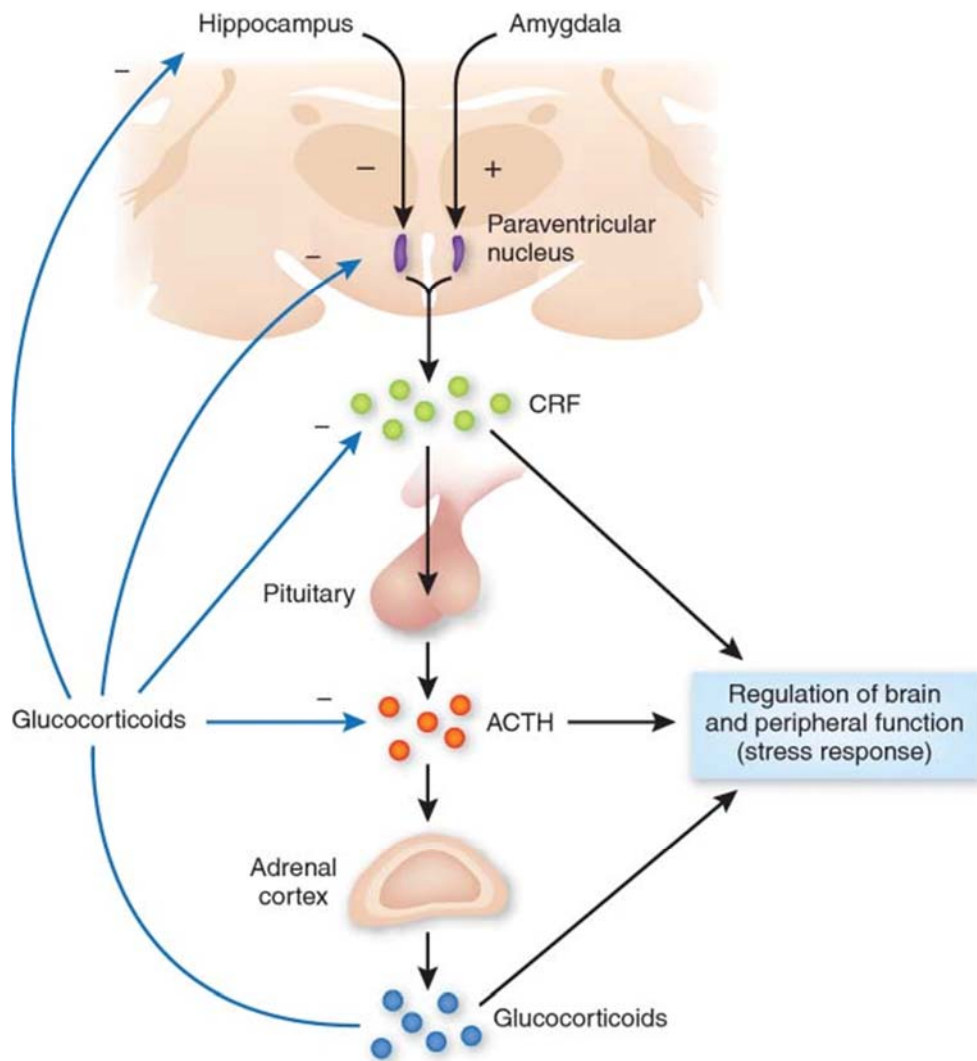


Figure 1-1. Hypothalamic-Pituitary-Adrenal Axis. In response to stressful stimuli the hypothalamus paraventricular nucleus (PVN) releases corticotrophin-releasing hormone (CRH), which targets the anterior pituitary gland. Adrenocorticotrophic hormone (ACTH) is then released into the circulation by the pituitary gland. In turn, the adrenal cortex secretes cortisol (in humans) or corticosterone (in rodents). Glucocorticoids bind to the receptors in the hippocampus providing the negative feedback to the HPA, whereas binding in the amygdala provides excitation to neurons in PVN enhancing the HPA activity. Figure adapted from (Hyman, 2009) under copyright license agreement #4007150059362.

(Dunn & Orr, 1984; Rubin, Mandell, & Crandall, 1966; Dunn & Whitener, 1986).

Interestingly, total hippocampectomy, hippocampal excitotoxic lesions, and hippocampal glucocorticoid receptor depletion can increase corticosterone (CORT) and ACTH production (Fendler, Karmos, & Teledy, 1961; Knigge & Hays, 1963; Knigge, 1961; Sapolsky et al., 1986).

Short-term HPA axis activation is critical for an organism's survival and homeostasis; however, sustained and excessive glucocorticoid production resulting from chronic stress is associated with negative feedback loop malfunction and the development of various pathologies. Prolonged HPA axis activation is associated with multiple disorders including sleep dysfunction, memory impairments, reduced sexual behavior, immune related disorders, and dysphoria (Lau et al., 2012; McEwen, 1998; McEwen, 2006). Chronic exposure to stress brings about pathological changes in the brain, particularly in the structures involved in the stress response, such as the PFC, hypothalamus, and hippocampus. For example, repeated exposure to high levels of glucocorticoids was reported to reduce adult neurogenesis, increase excitotoxicity and cell death, and alter dendritic and spine morphology (Dranovsky & Hen, 2006). Interestingly, numerous studies have demonstrated that hippocampal damage resulting from chronic stress is correlated with several behavioral changes including depression- and anxiety-like behaviors, and impaired learning and memory function in rodents (Daniel, 2002; Duman, Malberg, & Nakagawa, 2001; Duman, 2002; McEwen, 1999; McEwen & Magarinos, 2001; Nestler, Barrot, et al., 2002).

1.3. Depression

Major Depressive Disorder is a highly prevalent neuropsychiatric disorder, and it is estimated to affect 16.6% of the general population (Kessler & Wang, 2008). It is characterized by the persistence of one or two core symptoms: decreased mood (sad feelings)

and anhedonia (inability to experience pleasure); and several minor symptoms, which may include changes in weight and appetite, changes in sleep behaviour, fatigue (loss of energy), diminished ability to think and /or concentrate, feelings of worthlessness, and suicidal ideations. Major depression is diagnosed in accordance with the Diagnostic and Statistical Manual of Mental Disorders (DSM-V), which postulates that a diagnosis is made when the patient experiences one or more of the core symptoms and at least five of minor symptoms for greater than two weeks. Moreover, individuals suffering from depression have reduced social functioning and increased mortality rate (Nemeroff, 1998). Finally, proper diagnosis and successful treatment of this disorder are further complicated by the absence of biomarkers and its comorbidity with other neuropsychiatric conditions, such as anxiety, bipolar disorder, and posttraumatic stress disorder (Hirschfeld, 2001; Oquendo et al., 2003; Zimmerman et al., 2013).

It is widely acknowledged that stress is a major risk factor for depression. Evidence from numerous research lines supports the involvement of stress in depression. For example, individuals who experienced stressful environment in childhood have a sensitized HPA response (Essex, Klein, Cho, & Kalin, 2002), and have an increased likelihood of developing major depression as adults and adolescents (Espejo et al., 2007; Lenze, Xiong, & Sheline, 2008; McLaughlin et al., 2010). Likewise, patients suffering from depression often have hypercortisolemia, exhibit changes in cortisol rhythmicity (Sachar & Baron, 1979), and sensitized increases in cortisol release in response to stress (Belmaker & Agam, 2008; Burke, Davis, Otte, & Mohr, 2005; Checkley, 1996; De Kloet, Vreugdenhil, Oitzl, & Joëls, 1998; R M Sapolsky et al., 1984a). There is also evidence of elevated levels of CRH in cerebrospinal fluid and elevated urinary free cortisol in depressed individuals (Nestler, Barrot, et al., 2002; Southwick, Vythilingam, & Charney, 2005). Furthermore, individuals with Cushing's disease, a disorder characterized by chronically elevated levels of cortisol,

have unusually high rates of major depression (Sonino & Fava, 2001). Additionally, depressed individuals that did not show stabilization of ACTH and cortisol secretion after administration of dexamethasone (the synthetic glucocorticoid), also had a depressive episode relapse after antidepressant treatment (Nestler, Barrot, et al., 2002; Southwick et al., 2005). The involvement of stress in depression is further supported by research demonstrating that various classes of common antidepressants that modulate monoaminergic neurotransmission can also affect HPA axis function (Heuser et al., 1996; Linkowski et al., 1987; Schüle et al., 2009). Additionally, an antidepressant, metyrapone, which directly regulates cortisol production, is efficacious in treating major depression (O'Dwyer, Lightman, Marks, & Checkley, 1995). Taken together, clinical literature provides substantial support for the influence of a dysregulated stress response system in the development of major depression.

1.4. Recurrent Depression

Major depression is recognized as a highly recurrent and chronic condition. Over 70% of individuals suffering from depression experience more than one episode (Hollon et al., 2006; Solomon et al., 2000). Patients with recurrent depression have longer episodes and more severe symptomatology (Karp et al., 2004; Keller et al., 1992) and do not have a complete recovery during remissions (Judd et al., 1998, 2000).

According to the review by Hardeveld et al., the two primary predictors of recurrence are the number of previous depressive episodes and the presence of residual symptoms after recovery (Hardeveld, Spijker, De Graaf, Nolen, & Beekman, 2010). For example, with the increased number of depressive episodes, the number of prior episodes predicts future episodes. It is estimated that 60% of individuals who have had one depressive episode will have another, 70% of individuals with two depressive episodes will have a third, and 90% of individuals with three episodes will have a fourth episode (Kendler, Thornton, & Gardner,

2000). In contrast, demographic variables, i.e., sex, age, socioeconomic status, and marital status were not found to be associated with depression relapse and recurrence (Eaton et al., 1997; Gopinath, Katon, Russo, & Ludman, 2007; Kanai et al., 2003; Maj, Veltro, Pirozzi, Lobracc, & Magliano, 1992). However, some research studies do provide some evidence of influence of demographic factors on depression recurrence. For instance, Mueller et al. and Winokur et al. demonstrated that women had an increased risk of recurrence (Mueller et al., 1999; Winokur, Coryell, Keller, Endicott, & Akiskal, 1993). A greater risk of recurrence was observed among patients who were single (never been married).

Clinical literature suggests that the relationship between stress and depression changes over time. Although the initial depressive episode onset is strongly correlated with major life stressful events (Kendler et al., 1999; McLaughlin et al., 2010; Muscatell et al., 2009; Ormel et al., 2001; Parker et al., 2003), the subsequent episodes are rarely predicted by the major life stressors (Gonzales, Lewinsohn, & Clarke, 1985; Harkness, Monroe, Simons, & Thase, 1999; Kendler, Thornton, & Gardner, 2001; Kendler, Karkowski, & Prescott, 1999; Kendler et al., 2000). Two hypotheses have been put forward in the attempt to explain the changing relationship between stress and depression episode recurrence. The kindling hypothesis suggests that over the course of a lifetime of depressive episodes, the successive episodes become increasingly autonomous of stress and occur spontaneously (Monroe & Harkness, 2005; Post, 1992). This hypothesis is supported by several research studies demonstrating that major life stress is not a predictor of subsequent depression episodes (Gonzales et al., 1985; Harkness et al., 1999; Kendler et al., 2001; Kendler et al., 1999; Kendler et al., 2000). The stress sensitization hypothesis suggests that an individual's threshold of stress lowers with every successive episode of depression, therefore, the sensitivity to stress increases, leaving the depressed individual susceptible to depression relapse caused by minor stressors (Post, 1985). Several studies support this hypothesis

demonstrating that a relapse into a depressive episode is correlated with minor, but not major life stressors (Kendler et al., 1999; Ormel et al., 2001). Additionally, adolescent individuals were reported to have a depression relapse associated with both major and minor life stressors (Morris, Ciesla, & Garber, 2010). Furthermore, the preclinical literature also demonstrates support for the stress sensitization theory by reporting that animals pre-exposed to stress exhibit a sensitized release of catecholamines and elevated CORT levels following a subsequent stress re-exposure (Gresch, Sved, Zigmond, & Finlay, 1994; Grippo, Cushing, & Carter, 2007). Finally, findings from developmental research also demonstrate that animals stressed *in utero* or during adolescence develop depression-like symptoms in adulthood (Morley-Fletcher et al., 2003; Zurita, Martijena, Cuadra, Brandão, & Molina, 2000).

Both the kindling and stress sensitization theories of recurrent depression imply that certain changes at the biological level occur during the initial episode of depression, inducing a persistent change and increasing the susceptibility of the depressed individual to relapse into future episodes of depression. Interestingly, a longer course of depression (greater number and duration of episodes) is correlated with larger neurobiological changes, such as reduced volume of the hippocampus (Bremner et al., 2000; Sheline, Sanghavi, Mintun, & Gado, 1999; Sheline, Gado, & Kraemer, 2003). This observation further supports the idea that depression could be a progressive disorder, with increasingly larger neurobiological alterations leading to increasingly profound symptomatology and episode relapse.

Despite the significant impact and devastating consequences of depression on the patient and the public, the exact neurobiological mechanisms of depression etiology and its recurrent disease course remain unknown. Interestingly, cognitive-based therapeutic interventions, that have been designed specifically to reduce relapse or recurrence of major depressive disorder (Segal, Williams, & Teasdale, 2002), have shown some success. Research studies (Teasdale et al., 2000) and meta-analyses (Piet & Hougaard, 2011) report

that mindfulness-based cognitive therapy (MBCT) benefits patients with three or more depressive episodes in lowering the risk of relapse. Therefore, investigation of the neurobiological mechanisms of depression relapse and its effective prevention needs to be a high priority in mental health research.

1.5. Animal Models of Depression

Although clinical research has improved our understanding of risk factors, symptomatology, and neural correlates of depression, methods of clinical investigation are limited; the data are correlational and confounded with subject variability. Preclinical models are highly challenging, but they are essential tools for studying the etiology of mental disorders, the neurobiological changes associated with such disorders, biomarker development, and finding efficacious novel treatment programs. Animal models allow for subject homogeneity and control over experimental manipulation. Most psychiatric disorders, including major depression, are very heterogeneous syndromes, characterized by numerous symptoms that can be inferred with substantial limitations in animal preparations. It is virtually impossible to develop an animal model that reproduces a whole psychiatric syndrome; therefore, researchers typically focus on endophenotypes or discrete traits of the disorders (Gottesman & Gould, 2003). In depression preclinical research, a number of behavioral measures have been employed to identify a depression-like state in rodents (e.g., helplessness behaviors, anhedonia, weight loss, memory impairments, sleep disturbances, changes in sexual behavior), of which depression-like behavior (learned helplessness) and anhedonia are the most commonly used. Anhedonia (i.e., a lack of interest in pleasurable activity) is inferred by assessing an animal's preference for a sucrose solution over regular water. Healthy rodents typically prefer the sucrose solution, so a reduction in sucrose preference is considered to be indicative of anhedonic behavior (David et al., 2009; Gorzalka,

Hanson, Harrington, Killam, & Campbell-Meiklejohn, 2003; Gourley, Kiraly, Howell, Olausson, & Taylor, 2008). Rodent learned helplessness behavior can be measured in several ways, but the most common and reliable method is the use of the forced swim test (FST). In the FST, rats are forced to swim in a tank filled with water with no opportunity of escape. Depression-like behavior is inferred from longer time spent immobile (passive behavior) and shorter time spent swimming or climbing (active behaviors) (Lucki, 1997). Both the sucrose preference test (SPT) and the FST are thought to be valid tests for depression because various antidepressant treatment programs, including typical and atypical antidepressant drugs, electroconvulsive therapy (ECT), and rapid eye movement (REM) sleep deprivation reduce immobility in the FST (Armario, Gavalda, & Martí, 1988; Porsolt, Anton, Blavet, & Jalfre, 1978) and increase preference for sucrose (Willner, 1997). Interestingly, the clinical potency of antidepressants in humans is significantly correlated with their potency as tested in the FST (Borsini & Meli, 1988; Porsolt, Le Pichon, & Jalfre, 1977).

Specific criteria have been developed to judge the validity of the various animal models of human disorders: (1) face validity - the presence of the precipitating factors for developing the disorder, behavioral symptomatic similarities with the clinical disorder, common neurobiological changes; (2) predictive validity - therapeutic intervention results in similar changes that are seen in patient populations; and (3) construct validity - suggests that animal models should be defined so that a rational theory can be constructed to explain and test hypotheses regarding the etiology and pathogenesis of the disease (Geyer & Markou, 2002; McKinney & Bunney 1969; Willner, 2005). These criteria help to evaluate new models and compare existing models, although they do have several flaws (Nestler & Hyman, 2010). For example, the evaluation of construct validity is rather restricted, because our understanding of etiology of most psychiatric disorders is rudimentary. Additionally, the assessment of reversibility of the modeled symptoms with known drugs has limited value, as

many medications, such as monoamine-based antidepressants, have poor efficacy in a large subgroup of depressed patients (Berton & Nestler, 2006).

Several animal models have been utilized to investigate the etiology and pathogenesis of depression. Taking into consideration the well-characterized involvement of stress in depression, researchers have developed a number of stress-induced animal models of depression. The most common ones include chronic mild/unpredictable stress (CMS), repeated restraint stress, social defeat stress, repeated glucocorticoid exposure, and early-life stress (Cryan & Slattery, 2007). In the CMS model, rodents are subjected to various mild stressors (e.g., food/water deprivation, reversed light cycle, cage tilting, noise, cold stress) over a period of several weeks (Willner, 1997; Willner, 2005). Repeated restraint stress is one of the oldest models of depression used in research; it involves physically restraining an animal for a specific duration (usually hours) repeatedly over several days or weeks (Buynitsky & Mostofsky, 2009; Selye, 1976). Social defeat stress refers to a repeatedly (direct or indirect) exposing an animal to an aggressive and dominant animal (Hollis & Kabbaj, 2014). In early life stress models of depression, the animals are subjected to prenatal stress, maternal separation, or a combination of these stressors (Schmidt, Wang, & Meijer, 2011). Finally, the exogenous glucocorticoid exposure model involves the administration of glucocorticoids via pellet implantation, infusions, subcutaneous injections, or the drinking water (Nestler, Gould, & Manji, 2002; Sterner & Kalynchuk, 2010). Although some of these models have the advantages of producing more naturalistic stress (CMS, social defeat, and early life stress; Nestler, Gould, et al., 2002), many of the existing stress-induced models of depression lack reliability in producing robust and reliable increases in depression-like behavior. For example, conflicting results have been reported in several studies using CMS (Willner, 1997; Willner, 2005) and restraint stress (Gregus, Wintink, Davis, & Kalynchuk, 2005; Joo et al., 2009; Lussier, Caruncho, & Kalynchuk, 2009; Perrot-Sinal, Gregus,

Boudreau, & Kalynchuk, 2004; Regenthal, Koch, Köhler, Preiss, & Krügel, 2009; Veena, Srikumar, Raju, & Shankaranarayana Rao, 2009). The procedural variability (e.g., CMS), individual differences in HPA axis responses, and potential habituation effects (restraint stress) could account for inconsistencies across laboratories (Willner, 2005).

1.6. Exogenous CORT Administration Model of Depression

A large body of evidence demonstrates that exogenous CORT administration results in robust and reliable changes in depression-like and anhedonia-like behaviors (for a review, see Sterner & Kalynchuk, 2010). For example, rodents subjected to exogenous CORT (Brummelte, Pawluski, & Galea, 2006; David et al., 2009; Gourley et al., 2008; Gregus et al., 2005; Hill, Brotto, Lee, & Gorzalka, 2003; Johnson, Fournier, & Kalynchuk, 2006; Kalynchuk, Gregus, Boudreau, & Perrot-Sinal, 2004; Marks, Fournier, & Kalynchuk, 2009; Murray, Smith, & Hutson, 2008; Zhao, Ma, et al., 2008) display depression-like behaviors: a reduced latency to immobility, a reduced time spent swimming, and an increased time spent immobile in the FST. Our laboratory (Johnson, Fournier, & Kalynchuk, 2006) and others (Zhao, Ma, et al., 2008; Zhao, Shen, et al., 2008) have shown that CORT increases depression-like behavior in dose- (10, 20 and 40 mg/kg) and time-dependent (7, 14, and 21 days) manner (Lussier et al., 2013). Specifically, rats treated with 40 mg/kg CORT showed greater immobility than rats treated with CORT 20 mg/kg and CORT 10 mg/kg, with CORT 20 mg/kg group demonstrating intermediate immobility rate (Johnson, Fournier, & Kalynchuk, 2006). In contrast, a single injection of CORT was found to produce no effect on the FST behavior (Gregus et al., 2005). In addition, CORT was shown to have a time-dependent effect: rats treated with CORT demonstrate gradual increases in time spent immobile at 7, 14, and 21 days of treatment, reaching a statistically significant difference from control rats after 21 days of treatment (Lussier et al., 2013). Collectively, these findings

suggest that repeated CORT administration is a reliable method of inducing a depressive phenotype in rodents.

In addition to altering FST behavior, CORT can also produce other rodent behavioral and physiological changes that are similar to those seen in a human depression syndrome. For example, rats subjected to exogenous CORT often display impaired sexual behavior (Gorzalka, Brotto, & Hong, 1999; Gorzalka, Hanson, & Hong, 2001; Gorzalka & Hanson, 1998; Hanson & Gorzalka, 1999), decreased sucrose preference or intake (David et al., 2009; Gorzalka et al., 2003; Gourley et al., 2008), and inhibited grooming behaviors (David et al., 2009), all of which are anhedonic-like behaviors. Additionally, repeated CORT exposure can increase anxiety-like behaviors in rodents (David et al., 2009; Kalynchuk et al., 2004; Lee, Shim, Lee, Yang, & Hahm, 2009; Murray et al., 2008; Myers & Greenwood-Van Meerveld, 2007; Pêgo et al., 2008; Shepard, Barron, & Myers, 2000). Clinical depression is highly comorbid with anxiety disorders; therefore, the anxiety-like behaviors observed in the CORT model further improve its face validity (Hirschfeld, 2001). The physiological changes produced by CORT administration include decreased weight gain (Barr, Brotto, & Phillips, 2000; Brummelte et al., 2006; Bush, Middlemiss, Marsden, & Fone, 2003; Coburn-Litvak, Pothakos, Tata, McCloskey, & Anderson, 2003; Gregus et al., 2005; Johnson et al., 2006; Pêgo et al., 2008), dysregulated HPA axis functioning (Johnson et al., 2006), and reduced adrenal weight (Bush et al., 2003; Meijer et al., 1997; Murray et al., 2008). Importantly, the CORT-induced behavioral changes discussed above can be reversed by antidepressant treatment (David et al., 2009; Fenton et al., 2015), reinforcing the predictive validity of glucocorticoid exposure as a preclinical model of major depression. Finally, it is important to note that glucocorticoid administration models have an advantage of simulating exposure to stressful stimuli without the confounds associated with habituation effects and subject response variability (as seen in CMS and restraint stress models), ensuring that each animal

receives the same amount of glucocorticoids (Gregus et al., 2005; Grissom, Iyer, Vining, & Bhatnagar, 2007). Taken together, repeated CORT exposure offers a useful way to study the effects of glucocorticoids on brain and behavior in a preclinical setting.

1.7. Modeling Recurrent Depression in Animals

Despite the wealth of research using animal models of depression, very few studies have attempted to model the clinical course of the depression disorder. As discussed earlier, major depression is a chronic and recurrent disorder (Hollon et al., 2006; Kendler et al., 2000; Solomon et al., 2000). To our knowledge, only one study examined the sensitization of depression-like behavior using the CMS animal model of recurrent depression (Remus, Jamison, & Johnson, 2013). Remus and colleagues hypothesized that the experience of a prior depressive episode would sensitize rats to future depressive episodes. Indeed, rats exposed to CMS for 35 days, followed by a 20-day recovery period, and then re-exposed to the second session of CMS for 15 days, exhibited a more rapid (sensitized) decline in sucrose consumption compared with non-stressed control rats. The researchers interpreted these results as a sensitized depression-like behavior and a model of recurrent depression (Remus et al., 2013).

To better understand the neurobiological mechanisms underlying the recurrent nature of major depression, an animal model with repeated and cyclic stress treatment would be beneficial. This dissertation is, therefore, focused on developing a CORT model of recurrent depression and studying the effects of repeated and cyclic CORT administration on rodent depression-like behaviors and related neurobiological changes.

1.8. Hippocampal Anatomy and Circuitry

The hippocampal formation is located in the temporal lobe of the mammalian brain; it

comprises the hippocampus proper, or Cornu Ammonis (CA), dentate gyrus (DG), the subicular cortex (subiculum, presubiculum, and parasubiculum) and the entorhinal cortex (EC) (Amaral & Witter, 1989; Amaral, Dolorfo, & Alvarez-rojo, 1991).

The hippocampus proper has three main divisions: CA1, CA2 and CA3 (Figure 1-2). The stratum pyramidale is the principal cell layer in the CA subfields, consisting of pyramidal neurons (the primary excitatory cells in the hippocampus) and some interneurons projecting to adjacent pyramidal cells and interneurons in the CA. The stratum oriens is the next layer superficial to the stratum pyramidale; it houses inhibitory interneuron cell bodies as well as the basal dendrites of pyramidal neurons. Above the pyramidal layers are the stratum radiatum and stratum lacunosum-moleculare. The stratum radiatum consists of Shaffer collateral fibers projecting forward from CA3 to CA1 and interneuron cell bodies projecting to neighboring excitatory and inhibitory neurons. The stratum lacunosum-moleculare is the most peripheral layer in the hippocampus proper, where perforant path fibers form synapses at the apical dendrites of pyramidal cells (Amaral & Witter, 1989).

The DG is the major area of cortical input to the hippocampus. This region comprises three layers: the stratum granulosum (or granule cell layer, GCL), the polymorphic layer (or hilus), and the stratum moleculare (Amaral & Witter, 1989; Insausti & Amaral, 2004). A small area between the hilus and GCL is identified as the subgranular zone (SGZ), one of the two neurogenic sites in the mammalian brain. The GCL is the principal cell layer of the DG; it contains tightly packed excitatory granule neurons, extending apical processes into the molecular layer and axons into the hilus. The hilus consists of several types of neurons including inhibitory interneurons and excitatory mossy cells. The hilar pyramidal basket cells are the most studied interneurons in this region. In addition, the hilus contains other types of inhibitory interneurons innervating both excitatory and inhibitory cells in the DG (Freund & Buzsáki, 1996). The stratum moleculare is mainly comprised of dendritic processes of

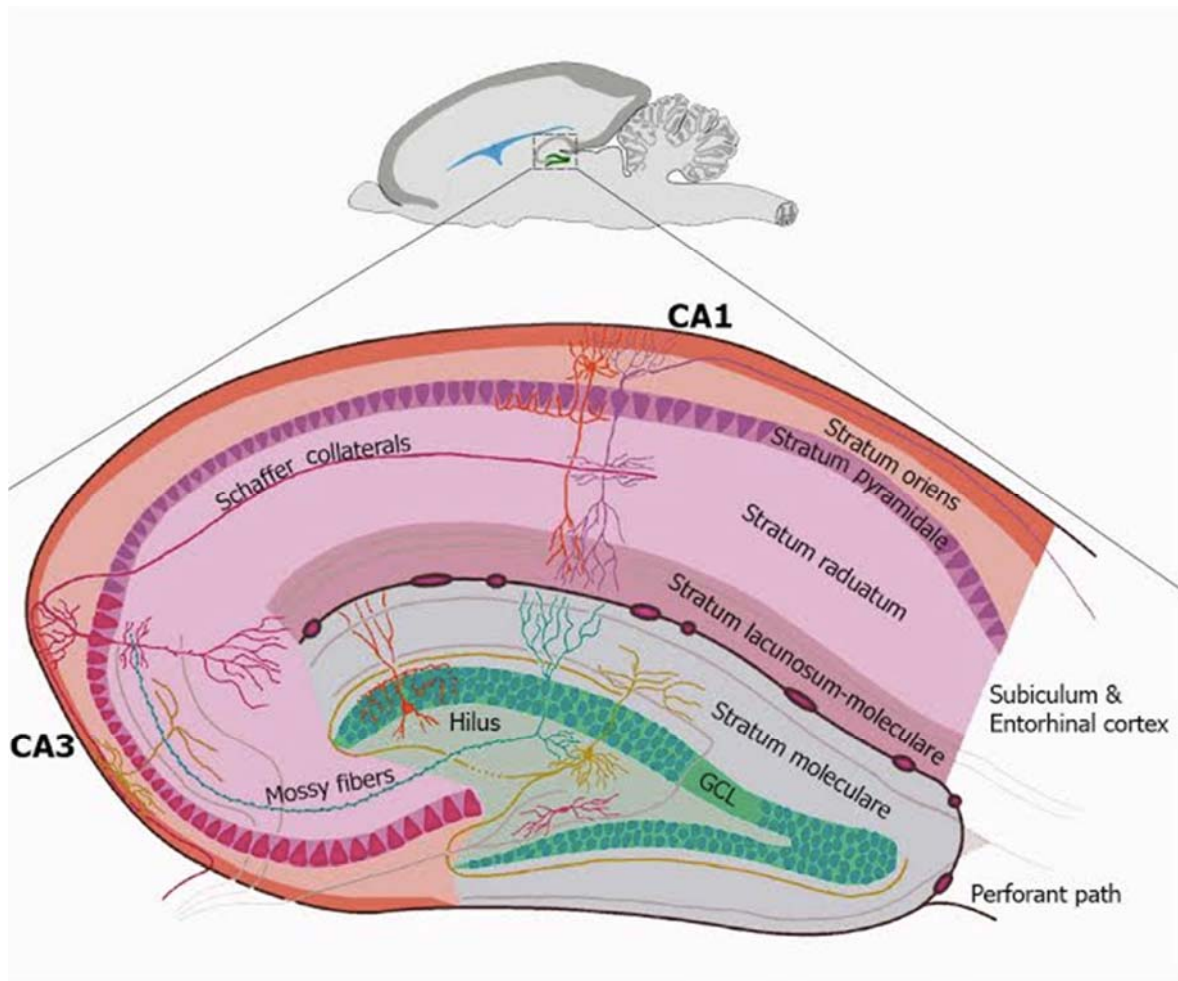


Figure 1-2. Schematic representation of rodent hippocampal formation and its circuitry. The image depicts laminar organization of the dentate gyrus (DG), Cornu Ammonis CA1 and CA3. The dentate gyrus is comprised of three layers: stratum moleculare, granule cell layer (GCL), and hilus. The stratum pyramidale consists of primarily cell bodies of pyramidal neurons. Just below the pyramidal cell layer is stratum oriens. Strata radiatum is located above the pyramidal cell layer and contains Schaffer collateral fibers, which are the primary projection from CA3 to CA1. Just above stratum radiatum is stratum lacunosum-moleculare, which contains synapses of perforant path fibers on dendrites of pyramidal cells. The tri-synaptic pathway: neurons in entorhinal cortex (EC) project to the DG via the perforant path, i.e., major hippocampal input; granule cells in DG give rise mossy fibers synapsing onto the pyramidal cells of CA3; axons of CA3 pyramidal neurons project to CA1 via the Schaffer collateral fibers; finally, axons of CA1 pyramidal neurons project to subiculum and EC, comprising the major hippocampal excitatory output. For details see text.

granule, basket, and hilar neurons; EC cell axons; inhibitory interneurons innervating granule neurons and adjacent inhibitory cells (Amaral & Witter, 1989; Freund & Buzsáki, 1996; Insausti & Amaral, 2004).

The intrahippocampal circuit is mainly unidirectional, in which the cortical input reaching the DG does so via the EC. Specifically, EC neurons have axons that project to the DG, forming the perforant path (the primary hippocampal input pathway). Then the excitatory granule cells of the DG form synapses via mossy fibers onto the cell bodies of pyramidal neurons in the CA3 subfield; in turn, the axons of CA3 pyramidal cells (or Schaffer collateral axons) project to the CA1 subfield, forming synaptic connections with adjacent dendrites of CA1 pyramidal cells. Finally, the axons of CA1 pyramidal neurons then provide input to the subiculum and EC, which are the primary excitatory output for the hippocampus (Amaral, Ishizuka, & Claiborne, 1990; Amaral & Witter, 1989; Insausti & Amaral, 2004). The synaptic connections in the perforant path, mossy fibers and Schaffer collaterals in the hippocampal network form the trisynaptic circuit (Felleman & Van Essen, 1991).

1.9. Adult Hippocampal Neurogenesis

Neurogenesis, or birth of new neurons, was initially believed to be an exclusively developmental process. However, in the 1960s Altman reported the first evidence that the adult brain has dividing cells that differentiate into cells with neuron-like morphology (Altman & Das, 1965; Altman, 1969). More evidence emerged later in support of adult neurogenesis in several species, such as rats (Altman & Das, 1965; Altman, 1969; Gould, Cameron, Daniels, Woolley, & McEwen, 1992), rabbits (Gueneau, Privat, Drouet, & Court, 1982), cats (Wyss & Sripanidkulchai, 1985), mice (Kempermann, Kuhn, & Gage, 1997a), non-human primates (Gould, Reeves, Fallah, et al., 1999; Gould, Reeves, Graziano, & Gross,

1999), and humans (Eriksson et al., 1998). It is well known today that mature neurons are not capable of division, however, in two distinct regions of adult brain, a population of stem cells can divide and differentiate into neurons or glial cells.

1.9.1. Stages of Neurogenesis

Neurogenesis is a process that with multiple stages: proliferation, differentiation, migration, maturation, and integration (Kempermann & Gage, 2000). Radial glial cells are the most commonly found stem cells in the adult mammalian brain and are typically referred to as type-1 cells. Radial glia have similar morphological and electrophysiological properties as astrocytes (Doetsch, 2003; Seri, García-Verdugo, McEwen, & Alvarez-Buylla, 2001), and they express glial fibrillary acidic protein (GFAP) and the neural stem cell marker nestin (Filippov et al., 2003; Fukuda et al., 2003). Radial glial cells generate three neuronal lineages: oligodendrocytes, astrocytes, and neurons. Importantly, the proliferation of type-1 cells is thought to be uninfluenced by environmental factors (Encinas & Enikolopov, 2008; Kronenberg et al., 2003).

Type-1 cells produce progenitor cells: type-2 and type-3. Type-2 cells, or neural progenitor cells (NPC) express nestin, but not GFAP, and have a high probability of being BrdU-positive, and therefore, highly proliferative (Filippov et al., 2003; Kronenberg et al., 2003). Type-2a and type-2b are two common subtypes of these cells. Type-2a cells express nestin, but not doublecortin (DCX), whereas type-2b cells express both nestin and DCX (Kronenberg et al., 2003). DCX expression signifies the commitment to neuronal differentiation and migration (Francis et al., 1999). DCX co-expresses with polysialylated form of the neural adhesion molecule (PSA-NCAM) (Von Bohlen Und Halbach, 2007). Newborn granule cells go through a temporary period of PSA-NCAM and DCX expression (Brandt et al., 2003; Rao & Shetty, 2004). Type-2b cells take on the neuronal determination

by expressing the neuron-specific nuclear protein (NeuN) and Prospero-homeobox-1 transcription factor (Prox1). At this stage of development, various environmental factors, including exercise (Praag, Christie, Sejnowski, & Gage, 1999; Praag, Shubert, Zhao, & Gage, 2005; Wu et al., 2008), environmental enrichment (Veena, Srikumar, Mahati, et al., 2009; Veena, Srikumar, Raju, et al., 2009), learning (Kee, Teixeira, Wang, & Frankland, 2007), stress (Brummelte & Galea, 2010; Duman et al., 2001; Gould, McEwen, Tanapat, Galea, & Fuchs, 1997; Pham, Nacher, Hof, & McEwen, 2003), and antidepressant treatment (Anacker, Zunszain, Carvalho, & Pariante, 2011; Kee et al., 2007; Perera et al., 2007) can alter neuronal progenitor cell survival. It was reported that the survival of progenitor cells can be affected by elevations in gamma-aminobutyric acid (GABA) levels (Ge et al., 2006), or the induction of N-methyl-D-aspartate (NMDA) receptor subunits NR2B and NR1 (Nácher et al., 2007), increasing the levels of vascular endothelial growth factor (VEGF) fibroblast growth factor-2 (FGF-2) (Greene, Banasr, Lee, Warner-Schmidt, & Duman, 2009; Palmer, Markakis, Willhoite, Safar, & Gage, 1999; Warner-Schmidt & Duman, 2007), and brain-derived neurotrophic factor (BDNF) (Huang & Reichardt, 2001).

Type-3 cells (commonly referred to as neuroblasts) express DCX but cease to express nestin (Von Bohlen Und Halbach, 2007). Cells in this stage radially migrate into the inner third of the GCL and begin exhibiting neuron-like morphology (Kempermann & Kronenberg, 2003). Type-3 cells exit the cell cycle with the transient expression of the calcium-binding protein calretinin (Brandt et al., 2003). These cells are also Prox1-positive throughout granule cell development; Prox1 is found in mature granule cells as well (Filippov et al., 2003). DCX- and calretinin-positive immature neurons start projecting apical dendrites forming synapses in the outer molecular layer, and axons – projecting toward the CA3 region. In this phase of maturation, immature neurons are favorably recruited for activation by external stimuli from learning processes (Kee et al., 2007) and environmental enrichment (Brown et

al., 2003; Kempermann, Kuhn, & Gage, 1997b) due to their plasticity, higher excitability, and reduced threshold for long-term potentiation (Ge et al., 2006; Ge, Yang, Hsu, Ming, & Song, 2007; Wang, Scott, & Wojtowicz, 2000). Finally, mature neurons expressing calbindin (instead of calretinin) and the neuronal nuclei (NeuN) protein display extended dendritic morphology and become fully integrated into the circuitry (Piatti, Espósito, & Schinder, 2006). DCX and PSA-NCAM are no longer expressed by these cells (Brown et al., 2003).

The recent acknowledgment of adult neurogenesis by the scientific community has given rise to multiple areas of research examining the potential roles of this process in various conditions, including depression.

1.10. Hippocampus, Neurogenesis and Depression

The hippocampus has become an extensively studied brain region in depression for several reasons. First, it was found that depressed individuals show reduced hippocampal volume (Bremner et al., 2000; Sheline, Wang, Gado, Csernansky, & Vannier, 1996; Sheline et al., 2003; Sapolsky, 2001) and that this decrease was correlated with the number depressive episodes, suggesting that patients suffering from a longer depression course exhibit smaller hippocampal volume (Frodl et al., 2006, 2008; Jaracz, 2008; Lorenzetti, Allen, Fornito, & Yücel, 2009; MacQueen et al., 2003). Second, the hippocampus expresses a high number of glucocorticoid and mineralocorticoid receptors, indicating that it is highly involved in regulating stress responses and HPA axis function, and therefore, it is particularly susceptible to stress-related changes (Arriza, Simerly, Swanson, & Evans, 1988; Chao et al., 1989; Herman et al., 2003; Herman, Ostrander, Mueller, & Figueiredo, 2005; Jacobson & Sapolsky, 1991; Sapolsky, Krey, & McEwen, 1984b). Interestingly, some evidence suggests that levels of cortisol are associated with reduced hippocampal volume in patients suffering from major depression (Lorenzetti et al., 2009; Pariante & Miller, 2001; Pariante et al., 2003;

Sheline, 2000). Third, studies using preclinical models of depression consistently reveal stress-induced decreases in hippocampal proliferation and survival of newborn neurons, as well as increases in dendritic atrophy in the hippocampus (Brummelte & Galea, 2010; David et al., 2009; Galea et al., 1997; Gould, Tanapat, McEwen, Flügge, & Fuchs, 1998; Magariños, Verdugo, & McEwen, 1997; Magariños, McEwen, Flügge, & Fuchs, 1996; Magariños & McEwen, 1995; Mayer et al., 2006; Murray et al., 2008; Nestler, Barrot, et al., 2002; Pham et al., 2003; Sapolsky, 2004; Watanabe, Gould, & McEwen, 1992). Finally, various antidepressant treatment programs, including antidepressant medication, electroconvulsive shock therapy, and exercise, were found to reverse the effects of prolonged stress on neurogenesis and hippocampal cell atrophy, as well as decrease depressive-like behaviors in rodents (Czeh et al., 2001; Malberg, Eisch, Nestler, & Duman, 2000; Malberg & Duman, 2003; Mayer et al., 2006; Praag, Kempermann, & Gage, 1999; Scott, Wojtowicz, & Burnham, 2000). Likewise, antidepressant treatment was reported to protect against hippocampal volume loss in depressed patients (Sheline et al., 2003).

The findings discussed above comprise evidence and support for the neurogenesis hypothesis of depression, which posits that major depressive symptomatology results from chronic stress through the mechanisms of decreased neurogenic capacity (Dranovsky & Hen, 2006; Duman et al., 2001; Jacobs, van Praag, & Gage, 2000). However, despite strong support for this hypothesis from a variety of research studies, the neurogenesis hypothesis of depression is challenged by the absence of direct evidence of decreased neurogenesis in human depression. For instance, no significant reduction in cell proliferation and no cell loss were found in post-mortem analyses of brain tissue from depressed individuals (Müller et al., 2001; Reif et al., 2006; Stockmeier et al., 2004). In addition, several preclinical reports demonstrated that ablation of neurogenesis, using irradiation (Santarelli et al., 2003; Surget et al., 2008) or genetic techniques (Zhao, Deng, & Gage, 2008), is not necessary to induce a

depressive phenotype. Considering this evidence, it is implausible to suggest that impaired hippocampal neurogenesis is the sole determinant of depression, however, the idea that deficient neurogenesis plays a significant role in the pathogenesis of depression is widely accepted. More research is needed to investigate the link between impaired neurogenesis and the specific symptoms of depression, as well as antidepressant properties of restored neurogenic function (Sahay & Hen, 2007). Additionally, there is evidence to suggest that the etiology of depression could be associated not only with deficits in cell proliferation and survival, but rather, with abnormalities of the surviving dentate granule cells, i.e., their altered morphology (Lussier et al., 2009), improper migration and circuit integration (Duan et al., 2007), as well as abnormal transcriptional and molecular regulation (Duman & Monteggia, 2006; Nestler, Barrot, et al., 2002). Moreover, these abnormalities can potentially accumulate, rendering the individual vulnerable to other life events. For example, it was shown that repeatedly stressed rats displaying dendritic retraction within the CA3 region of hippocampus also exhibited exacerbated hippocampal damage following an excitotoxin metabolic challenge, suggesting that stress compromises the ability of hippocampus to resist the metabolic challenge and other adverse environments (Conrad, Jackson, & Wise, 2004). These observations are promising but incomplete; therefore, more research is needed to explore the cellular and molecular mechanisms promoting impaired hippocampal plasticity and depressed symptomatology.

1.11. Repressor Element-1 Silencing Transcription Factor (REST) and Depression

Long-term molecular changes and alterations in transcriptional regulation have been implicated in mood disorders and antidepressant action (Coyle & Duman, 2003; Duman & Monteggia, 2006; Manji et al., 1999; Nestler, Barrot, et al., 2002). Repressor element-1 silencing transcription factor (REST), also referred to as neuron-restrictive silencing factor

(NRSF), is a modular zinc finger protein that contains two independent repression domains at the N- and C-terminals of the molecule (Chong et al., 1995; Schoenherr & Anderson, 1995; Tapia-Ramírez, Eggen, Peral-Rubio, Toledo-Aral, & Mandel, 1997). Upon binding to DNA, REST works as an assembling frame, positioning its complexes, such as histone deacetylases and the demethylase LSD1. The resulting complexes allow repression of the transcription of large numbers of target genes by modifying critical sites of their histones (Ballas & Mandel, 2005; Chong et al., 1995; Huang, Myers, & Dingledine, 1999; Ooi & Wood, 2007; Schoenherr & Anderson, 1995). Initially, REST was known as a repressor of neuronal (RE-1 containing) genes during embryonic development only, serving as a switch that aids differentiation between neuronal and non-neuronal cell types. REST was thought to be down-regulated once terminal neuronal differentiation occurred (Ballas, Grunseich, Lu, Speh, & Mandel, 2005; Chong et al., 1995; Kraner, Chong, Tsay, & Mandel, 1992; Mori, Schoenherr, Vandenberg, & Anderson, 1992; Schoenherr & Anderson, 1995). However, recent evidence determined that the RE-1 sequence is a subset of a large number of REST-binding motifs (Bernstein et al., 2012; Johnson, Mortazavi, Myers, & Wold, 2007; Otto et al., 2007). Consequentially, a larger number of REST target genes have been discovered, including target genes coding for various mRNAs, microRNAs, short hairpin RNAs, and long noncoding RNAs (Johnson et al., 2009; Rossbach, 2011; Volvert et al., 2014). REST induction in mature neurons is a subject of plasticity. This is evidenced by observations that adult hippocampal granule and pyramidal neurons express high levels of REST (Calderone et al., 2003; Gao et al., 2011; Kuwabara, Hsieh, Nakashima, Taira, & Gage, 2004; Palm, Belluardo, Metsis, & Timmusk, 1998; Sun et al., 2005), that adult neurons upregulate REST and specific target genes in response to ischemia or seizures (Calderone et al., 2003; Jessberger et al., 2007; Palm et al., 1998), and that increased REST expression was reported in normal aging in human cortical and hippocampal neurons (Lu et al., 2014). In addition,

REST and its target genes play a role in adult neurogenesis and synaptic plasticity (Ballas et al., 2005; Westbrook et al., 2008, Schoenherr & Anderson, 1995).

Dysregulation of REST has been associated with several pathological conditions, including Down's syndrome (Bahn et al., 2002), Alzheimer's disease (Lu et al., 2014; Okazaki et al., 1995), Huntington's disease (Zuccato et al., 2007), ischemic insults (Calderone et al., 2003), epilepsy (Garriga-Canut et al., 2006; Hu et al., 2011), schizophrenia (Loe-Mie et al., 2010), and Parkinson's disease (Siegel, Saba, & Schratt, 2011; Yu et al., 2013). However, the potential role of REST in the pathogenesis of mood disorders remains unknown. A recent study reported that REST targets several genes that are known to be largely implicated in depression (Duman & Monteggia, 2006; Lemonde et al., 2003; Nestler, Barrot, et al., 2002); these genes include CRH, the serotonin (5-HT) 1A receptor, and BDNF (Otto et al., 2007). In addition, REST was found to participate in transcriptional regulation of cortisol/corticosterone synthesis (Somekawa et al., 2009). Further evidence of REST involvement in stress signaling comes from a study demonstrating that a variety of stressors are able induce the expression of REST in neuronal cultures, and that the increase of neuronal REST levels induces the expression of stress response genes (Lu et al., 2014). Finally, the repressive activity of REST is accompanied by a recruitment of histone deacetylases and chromatin-remodelling enzymes, which are also known to be altered in major depression (Schroeder, Lin, Crusio, & Akbarian, 2007; Tsankova et al., 2006).

A recent study on patients with major depressive and bipolar disorder showed that REST mRNA levels were decreased, whereas CRH, adenylate cyclase 5, and the tumor necrosis factor mRNA were increased in peripheral blood cells of patients (Otsuki et al., 2010). Interestingly, this reduction of REST mRNA and increases of CRH, adenylate cyclase 5, and the tumor necrosis factor mRNA were found in patients in a current depressive, but not a remissive state, suggesting that these alterations are state-dependent and could be associated

with depression pathophysiology (Otsuki et al., 2010). To our knowledge, the only study investigating brain REST expression in an animal model of depression was conducted by Uchida and colleagues (Uchida et al., 2010). These researchers reported that rats subjected to early-life stress and restraint stress in adulthood showed increased expression of REST4 (a splice variant of REST) in medial PFC (mPFC). Interestingly, REST4 overexpression in the mPFC of neonatal mice, but not adult mice, increased behavioral vulnerability to repeated restraint stress (Uchida et al., 2010). Collectively, these reports suggest that REST could play an important role in the regulation of stress vulnerability, neurogenesis, and the pathogenesis of mood disorders. Therefore, further investigation of how REST is altered in recurrent depression would help elucidate potential mechanisms of depression episode relapse and open new avenues for novel pharmacological intervention.

1.12. Inflammation, Stress, and Depression

Growing evidence implicates immune system activation in the etiology and pathophysiology of depression. Clinical studies and meta-analyses repeatedly report that patients with major depression have elevated concentrations of cytokines in the plasma and cerebrospinal fluid, in the absence of any physical illness or infection (Alesci et al., 2005; Dahl et al., 2014; Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008; Kern et al., 2014; Dowlati et al., 2010; Howren, Lamkin, & Suls, 2009). Moreover, a reduction of IL-1 and IL-6 levels was associated with alleviation of depressive symptoms in response to antidepressant treatment in depressed patients (Hannestad, DellaGioia, & Bloch, 2011). In addition, several depressive symptoms, such as guilt and suicidal ideation, are strongly associated with plasma IL-6 levels (Alesci et al., 2005; O'Donovan et al., 2013). Interestingly, cancer patients treated with therapy involving cytokines exhibit depressive symptoms that can be relieved with antidepressant medication (Capuron et al., 2002).

Preclinical research supports clinical observations and has revealed increased cytokine content in both brain and plasma of animals with depression-like behavior (Golovatscka, Ennes, Mayer, & Bradesi, 2012; Grippo, Francis, Beltz, Felder, & Johnson, 2005; Yirmiya & Goshen, 2011; You et al., 2011). Systemic and central treatment with proinflammatory cytokines, such as IL-1 or tumour necrosis factor-alpha (TNF-alpha) and lipopolysaccharide (LPS) were found to induce sickness behavior and/or depression-like behavior in animals dose-dependently (Dantzer, 2004; Dunn & Swiergiel, 2005; Henry et al., 2008; Laugeray et al., 2011). For example, mice administered with IL-1beta (100, 300 and 1,000 ng/mouse) and subsequently tested in the tail suspension test (TST) and forced swim test (FST) showed dose-dependent increases in the time spent immobile in both TST and FST, statistically significant increases were found with higher doses only (Dunn & Swiergiel, 2005). Interestingly, knockout mice lacking cytokines or cytokine receptors (e.g., interleukin-6 (IL-6) or interleukin-1 (IL-1) type 1 receptor) do not show stress-induced depression-like behavior (Chourbaji et al., 2006). In addition, it has been demonstrated that acute and chronic stress (inescapable shock, restraint), as well as glucocorticoid administration, can increase cytokine mRNA and protein levels in the brain and plasma (Blandino et al., 2009; Bowers, Bilbo, Dhabhar, & Nelson, 2008; Deak et al., 2005; Porterfield, Gabella, Simmons, & Johnson, 2012; Sorrells, Caso, Munhoz, & Sapolsky, 2009).

It is important to note that glucocorticoids are conventionally known to produce anti-inflammatory effects. For example, glucocorticoids are used pharmacologically to control post-surgery inflammation, as well as to treat asthma, allergies, and autoimmune diseases (Hockey, Leslie, & Williams, 2009), as they reliably reduce the expression of proinflammatory cytokines including IL-1beta, IL-6, and TNF-alpha and increase levels of anti-inflammatory mediators including IL-10 and transforming growth factor-beta (TGF-b) (Correale, Arias, & Gilmore, 1998; Gayo et al., 1998). An activated HPA axis can exert

robust immunosuppressing effects by inhibiting the activity of the key inflammatory transcription factor, nuclear light chain kappa (NF- κ B) (Lee et al., 1988; Silverman & Sternberg, 2012; Zuckerman, Shellhaas, & Butler, 1989). In contrast, as discussed earlier, major depression is strongly associated with significant elevations of both cortisol and proinflammatory cytokines, presenting a paradox of simultaneous overactivity of the immune and endocrine systems. Two theories have been put forward to explain this paradox: one theory postulates that the immune system becomes overactive as a result of a developed glucocorticoid resistance characterized by reduced glucocorticoid signaling, and the second theory discusses both pro- and anti-inflammatory effects of glucocorticoids under specific circumstances (Horowitz & Zunszain, 2015). Under the first theory, the initial exposure to CORT may desensitize or reduce the number of glucocorticoid receptors resulting in a decrease in intracellular glucocorticoid signaling and a subsequent decrease in the ability of glucocorticoids to inhibit the immune system. This hypothesis has found support from both clinical and preclinical research demonstrating glucocorticoid resistance (less transcriptional signaling for each cortisol molecule) co-occurring with elevations in pro-inflammatory cytokines in depressed individuals (Bauer et al., 2003; Carvalho et al., 2008; Fitzgerald et al., 2006; Humphreys, Schlesinger, Lopez, & Araya, 2006; Landmann, Schaub, Link, & Wacker, 1997; Maes, Scharpé, Meltzer, & Cosyns, 1993) and in stress-induced models of depression (Avitsur, Powell, Padgett, & Sheridan, 2009; Sheridan, Stark, Avitsur, & Padgett, 2000). The second proposed theory emphasizes that glucocorticoids are not invariably anti-inflammatory, but rather that they can be both pro- and anti-inflammatory under certain conditions (Frank, Watkins, & Maier, 2013; Sorrells et al., 2009). In particular, preclinical studies report that exposure to glucocorticoids (or stress) after an inflammatory event results in immunosuppression (Goujon et al., 1995). In contrast, exposure to glucocorticoids (or stress) before an inflammatory insult enhances the immune system response (de Pablos et al.,

2006; Espinosa-Oliva et al., 2011; Frank, Miguel, Watkins, & Maier, 2010; Johnson et al., 2002; Munhoz et al., 2006; Munhoz, Sorrells, Caso, Scavone, & Sapolsky, 2010). Similarly, preliminary studies demonstrated that administration of hydrocortisone simultaneously with LPS stimulation in healthy humans abolished the elevation of TNF-alpha, whereas hydrocortisone administered 12 to 144 hours before LPS resulted in significant increases in IL-6 and TNF-alpha (Barber et al., 1993). Finally, *in vitro* studies provide further support for pro-inflammatory effects of glucocorticoids by reporting enhanced activity and cytokine release of rodent macrophages in response to inflammatory stimulation after glucocorticoid pre-treatment (Smyth et al., 2004). Collectively, these data emphasize the complex relationship between the endocrine and immune systems; therefore further research focused on depression models integrating both systems would help clarify the mechanisms of depression pathogenesis.

1.13. Microglia

Microglia are immune cells that belong to the mononuclear phagocyte system (Walker, Nilsson, & Jones, 2013a). Newborn microglial cells have an amoeboid shape and no processes; however as the cells mature, microglia change their morphological phenotype, so that they become characterized by a small soma and numerous fine ramifications (Butovsky et al., 2014; Erbilich, Zhu, Etgen, Dobrenis, & Pollard, 2011; Kierdorf et al., 2013; Smith et al., 2013). Microglia are a major source of centrally released cytokines and other inflammatory mediators, however they are known to play an important role not only in the innate immune response but also in brain development, maintenance and repair (Michell-Robinson et al., 2015).

Depending on the circumstances, microglial cells have a very dynamic morphology and function (see Figure 1-3). In the absence of pathological conditions, microglia are in a

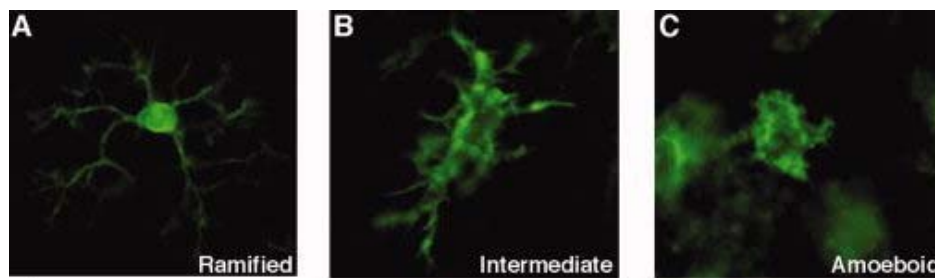


Figure 1-3. Dynamic morphological changes in a microglia cell. Panels A-C: photomicrographs showing examples of microglia classified into four different phenotypes: ramified, intermediate, and amoeboid. Figure adapted from (Thored et al., 2009) under copyright license agreement #4007161322200.

quiescent or “resting” state, constantly gauging the surrounding environment by spreading and retracting their motile branches. In doing this, microglia are able to communicate with surrounding cells, survey their territory, identify signals that demand a response, and maintain homeostasis (Davalos et al., 2005; Hanisch & Kettenmann, 2007; Kettenmann, Hanisch, Noda, & Verkhratsky, 2011; Lull & Block, 2010; Nimmerjahn, Kirchhoff, & Helmchen, 2005). Upon detection of potentially harmful signals (e.g., neuronal damage), microglia can rapidly acquire a reactive profile, which typically involves changes to the de-ramified morphological form. Such activated microglia also exhibit changes in cell surface markers (e.g., upregulated cluster of differentiation molecule 68 (CD68) and major histocompatibility complex II proteins), and they can proliferate, migrate to sites of damage, release pro-inflammatory cytokines, recruit and activate other cells, and phagocytose (Davalos et al., 2005; Johnson et al., 2002; Lynch, 2009; Streit & Kreutzberg, 1987). The morphological changes of activated microglia are characterized by a swollen cell body, thickened and shortened ramifications, and ultimately, in the phagocytic stage, an amoeboid-resembling shape (Kettenmann et al., 2011).

Microglial cells seem to be most densely populated in neurogenic niches such as the hippocampus (Lawson, Perry, Dri, & Gordon, 1990). Importantly, emerging evidence suggests that microglia can play an important role in hippocampal neurogenesis (Butovsky et al., 2014; Sierra et al., 2010), neural plasticity (Butovsky et al., 2014; Rogers et al., 2011), and synaptic maturation (Paolicelli et al., 2011; Zhan et al., 2014). In addition, microglia are involved in producing a number of growth factors, such as insulin-like growth factor 1 (IGF-1) and BDNF, which are important for neurogenesis as well (Butovsky et al., 2006; Coull et al., 2005; Sierra et al., 2010; Zhang et al., 2014). Finally, findings from several studies show that microglia are capable of making frequent contacts with neurons at presynaptic boutons, postsynaptic spines, and the synaptic cleft, and by doing this they participate in synapse

pruning, and therefore may contribute to the organization of neural circuitry (Paolicelli et al., 2011; Tremblay, Lowery, & Majewska, 2010; Wake, Moorhouse, Jinno, Kohsaka, & Nabekura, 2009).

1.14. Microglia, Stress, and Depression

A growing body of evidence indicates that neuroinflammatory processes are triggered not only in response to immune challenges but also in response to psychological and physiological stress that is not associated with injuries and infection (Blandino et al., 2009; Bowers et al., 2008; Deak et al., 2005; Porterfield et al., 2012; Sorrells et al., 2009). Glucocorticoid and mineralocorticoid receptors are highly expressed by microglial cells (Tanaka et al., 1997), and activation of microglia has been implicated in modulating the effects of stress (Inbal Goshen & Yirmiya, 2009). For example, exposure to acute stressful stimuli was found to induce microglial morphological (i.e., enlarged soma and thick short processes) (Sugama, Fujita, Hashimoto, & Conti, 2007) and functional activation (Frank, Baratta, Sprunger, Watkins, & Maier, 2007), whereas the microglial inhibitor minocycline was shown to abolish stress-induced hypothalamic IL-1 release (Blandino et al., 2009). Studies using repeated stress or glucocorticoid administration have revealed conflicting findings, suggesting that glucocorticoids can have both inhibitory and stimulatory effects on microglia (Walker, Nilsson, & Jones, 2013b). For instance, anti-glucocorticoid agents (e.g., RU486, metyrapone) or adrenalectomy both inhibit stress-induced microglial proliferation (Frank et al., 2007; Nair & Bonneau, 2006). Furthermore, it was shown that dexamethasone administration reduced ionized calcium-binding adapter molecule-1 (Iba-1) expression (Zhang, Zhang, Artelt, Burnet, & Schluesener, 2007), decreased CD68 expression (Hinkerohe et al., 2010) and reduced nitric oxide and pro-inflammatory cytokine production by microglial cell culture after LPS stimulation (Graber, Hickey, Stommel, & Harris, 2012).

In contrast, repeated stress in rodents was reported to increase the number of microglia, induce Iba-1 immunoreactivity in several stress-responsive brain regions (Nair & Bonneau, 2006; Tynan et al., 2010a), and alter microglial morphology (Wohleb et al., 2011). The ability of CORT itself to produce both stimulatory and suppressive effects on microglia has been investigated in a study, which found that when exogenous CORT treatment preceded LPS stimulation, the subsequent microglial immune response was enhanced. However, when CORT was delivered one hour after the LPS challenge, the pro-inflammatory response of microglia was inhibited (Frank et al., 2010). Collectively, these findings further emphasize the complex temporal relationship between glucocorticoids and immune responses, and they also highlight the critical need for integral investigation of these systems in the search for the mechanisms underlying psychiatric disorders, such as depression.

In addition to microglial involvement in stress, several lines of evidence suggest that microglia may play a significant role in depression. First, activation of microglia was reported in some depressed and suicidal individuals (Bayer, Buslei, Havas, & Falkai, 1999; Steiner et al., 2008). Second, as earlier discussed, depressed patients exhibit elevated CSF levels of pro-inflammatory cytokines, which are thought to be released by brain microglia (Dowlati et al., 2010; Howren et al., 2009). Third, depression is comorbid with several neurodegenerative and neurological diseases, such as Alzheimer's (Cameron & Landreth, 2010; Evans et al., 2005), which are characterized by profound microglial alterations (Kettenmann et al., 2011). Fourth, cancer and hepatitis C patients receiving interferon- α immunotherapy, as well as healthy individuals experimentally treated with LPS, develop depressive symptoms (Krabbe et al., 2005; Raison, Capuron, & Miller, 2006). Finally, antidepressant medications are known to regulate hippocampal neurogenesis and microglial activity (Hashioka et al., 2007; Malberg et al., 2000; Santarelli et al., 2003; Tynan et al.,

2012) and microglia, in turn, are important regulators of neurogenesis (Ek Dahl, Kokaia, & Lindvall, 2009; Ek Dahl, Claasen, Bonde, Kokaia, & Lindvall, 2003).

Several methods are available for visualizing microglial reactivity in a brain. For example, in depressed individuals, in vivo positron emission tomography (PET) technique is commonly used. The translocator protein density, which is elevated in reactive microglia, can be measured using PET distribution volume. Depressed individuals were found to have higher translocator protein density in the PFC, anterior cingulate cortex, and insula (Setiawan et al., 2015). In the animal models of depression, analysis of microglia cell surface markers is more feasible. In the activated microglia, upregulation of several markers is observed, such as antigen presentation molecules (CD68, CD80, CD86, and CD40) and major histocompatibility complex II proteins (Davalos et al., 2005; Johnson et al., 2002). In addition, often microglial morphology is examined to confirm dynamic changes in cells and to detect a potential “primed” state of microglia (susceptible to a secondary inflammatory insult). Such analyses warrant the use of markers that are constitutively expressed in microglia, i.e. CD11b, Iba-1, which are commonly used in depression preclinical research (Kreisel et al., 2014; Lehmann, Cooper, Maric, & Herkenham, 2016; Pan, Chen, Zhang, & Kong, 2014; Wohleb et al., 2012).

Taken together, this evidence strongly supports the involvement of microglia in the pathogenesis of depression, and suggests that further examination into microglial alterations affecting immune and stress responses may be of importance for understanding and treating disorders characterized by dysregulations of both endocrine and immune systems.

1.15. Goals and objectives

The general aim of the research presented in this dissertation is to develop and examine an animal model of recurrent depression using repeated and cyclic administration of glucocorticoids. Specifically, I have tested how pre-exposure to exogenous CORT affects rat

behavioral vulnerability to subsequent CORT re-exposures. These effects were examined using repeated and non-repeated behavioral testing strategies. Further, I have investigated how repeated and cyclic glucocorticoid treatment impacts adult neurogenesis and REST expression within the rat hippocampus. Finally, I have done a preliminary examination into the role of repeated and cyclic stress on microglia morphology and activation in the hippocampus.

Experiment 1. Repeated CORT administration is known to increase depression-like behavior in one of the most commonly used tests of rodent helplessness behavior, the FST (Nestler, Gould, et al., 2002). However, the majority of previous research has examined the behavioral changes after only one cycle (e.g. three weeks) of repeated exposure to stress. Taking into consideration the recurrent nature of depressive disorders (Hollon et al., 2006; Solomon et al., 2000), it is highly important to incorporate the depression disease course into stress-induced animal models of depression. To address this gap in the preclinical research, I examined the effects of two cycles of daily CORT injections (21 days), each followed by injection-free recovery periods (21 days), in male rats using repeated FST behavior testing in the middle (day 10) and in the end (day 22) of each stress cycle, and at the end of each recovery period.

Hypothesis: The effects of repeated and cyclic CORT injections were expected to be more pronounced during the second CORT cycle. In particular, I predicted that it would require fewer CORT injections (10 days) in treatment cycle 2 to produce increases in depression-like behavior in rats that were pre-exposed to CORT and allowed to recover.

Experiment 2. After identifying that repeated and cyclic CORT treatment produced progressively larger changes in rodent depression-like behavior, it was important to reproduce these effects in a model using naïve rats and a non-repeated behavioral testing strategy. This model would control for potential confounding effects of repetitive FST task

learning in the previous experiment. Additionally, it would be critical to investigate whether the aggravating effects of multiple CORT cycles extend to other behaviors associated with a depressive phenotype (i.e. anhedonia). Furthermore, our laboratory has previously shown that one cycle of repeated CORT treatment decreases neurogenesis and dentate granule cell maturation (Lussier et al., 2009). Clinical evidence suggests that depression could be a progressive disorder, as hippocampal volumetric reductions were found to be strongly associated with the number and duration of depressive episodes (Bremner et al., 2000; Sheline, Sanghavi, Mintun, & Gado, 1999; Sheline, Gado, & Kraemer, 2003). However, it is not known how multiple cycles of glucocorticoid exposure impact hippocampal plasticity, such as neurogenesis and neuronal maturation. Finally, considering the emerging evidence of aberrant brain transcriptional regulation in depression and other psychiatric disorders, examination of hippocampal REST protein expression alterations after one or several CORT exposures was of great interest.

Hypotheses: I expected that the findings of behavioral sensitization during CORT re-exposure in Experiment 1 would be replicated in Experiment 2 using non-repeated behavioral testing of naïve rats. Specifically, I hypothesized that rats pre-treated with CORT injections would display greater depression-like and anhedonia behaviors than rats exposed to CORT only once. I also predicted that shorter periods of CORT injections would increase depression-like and anhedonia behaviors in rats that experienced previous cycles of CORT exposure. In contrast, I expected no changes in general exploratory behavior in the open field test between groups, as CORT is known to have few effects of rat locomotion (Marks et al., 2009). Additionally, with every subsequent re-exposure to CORT, I expected that rats would have accumulative deficits in rates of neurogenesis and dentate granule cell maturation paralleled with progressive worsening of depression-like behaviors. Finally, previous reports suggest that REST plays critical roles in neurogenesis, stress, and depression (Ballas et al.,

2005; Otsuki et al., 2010; Uchida et al., 2010; Westbrook et al., 2008), therefore I hypothesized a progressive loss of hippocampal REST levels with multiple cycles of CORT treatment.

Experiment 3. A wealth of research demonstrates a substantial involvement of neuroinflammatory processes in depression. Considering the existing complex relationship between the endocrine and immune systems, as well as emerging evidence of significant impact of microglial cells on both brain immune response and hippocampal neurogenesis, I examined changes in hippocampal microglia in the CORT model of recurrent depression. To address this question, I analyzed changes in microglial immunoreactivity and morphology after one or three cycles of CORT exposure. Combining two existing hypotheses of depression (inflammatory and neurogenesis theories) allows for a more integrated approach and closer examination of underlying mechanisms that promote the development and progression of depression over time.

Hypothesis: I expected that one cycle of CORT would result in activation of microglial cells demonstrating a reactive phenotype of cell morphology: enlarged soma; fewer, thicker, and shorter processes. Moreover, I hypothesized that with additional cycles of CORT treatment, microglia cells would show potentiated effects in these morphological changes.

CHAPTER 2

Cyclical Corticosterone Administration as an Animal Model of Recurrent Depression: Results of Repeated Forced Swim Testing

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Abstract

Stress is a significant risk factor for depression. Many animal models of depression with stress exposure have been developed. However, almost without exception, stress-induced models of depression focus on reproducing single incidences of depression endophenotypes (e.g., anhedonia, negative mood bias) and therefore, they do not provide any means to understand the typical cycling of mood observed in most patients with depression. In the current study, we attempted to recapitulate the cyclical disease course of depression in a rodent model. Rats were treated with 20 or 40 mg/kg corticosterone (CORT), or vehicle for two cycles (21 days each), followed by a 21-day recovery period. Depression-like behavior was assessed via repeated forced swim tests (FSTs) in the middle and at the end of each CORT treatment and at the end of each recovery period. Our results demonstrate that repeated treatment with CORT for two cycles produced increasingly greater effects on depression-like behavior. In the 1st cycle, CORT increased the time spent immobile and decreased the time spent swimming in the FST after 21 days of treatment. These behaviors then normalized after the recovery. In the 2nd cycle, however, we observed an early manifestation of depression-like behavior after only ten days of CORT treatment, as well as diminished recovery with each subsequent cycle of treatment, suggesting sensitization of the depression-like symptoms in rats. Potential confounding effects of aging, body weight, and repetitive behavioral testing should be considered in the interpretation of sensitization of rat depressive-like behavior. The proposed model of recurrent depression provides stronger face validity as it mimics several aspects of human depression, including the phenomenon of episode recurrence seen in more than 70% of patients with depression.

2.1. Introduction

Depression is a serious psychiatric disorder and a profound public health concern. The complex structure of human depression and the subjective nature of its symptoms have made the development of animal models extremely challenging.

Exposure to stress has a strong impact on the manifestation of depression. A large body of experimental and clinical data suggests that stress is a major risk factor in the development of depression (Kendler et al., 1999; Mazure, 1998). For example, the initial depressive episodes are often preceded by stressful or traumatic life events (Keller, Schatzberg, & Maj, 2007). One of the key endocrine changes in depressed individuals is a dysregulation of the HPA axis (Nestler, Barrot, et al., 2002). Patients with depression often exhibit cortisol hypersecretion and disrupted cortisol rhythmicity (Burke et al., 2005; Sachar & Baron, 1979), which can be normalized with antidepressant treatment (Holsboer, 2001). Also, patients with Cushing's disease, a disorder characterized by chronically high levels of cortisol, show unusually high rates of depression (Sonino & Fava, 2001). Interestingly, preclinical research reveals similar trends: high levels of glucocorticoids have been shown to increase depression-like behaviors in rodents (i.e. increased immobility time in forced swim and tail suspension tests, and decreased sucrose preference). In addition, repeated exposure to glucocorticoids has been demonstrated to have deleterious effects on neurochemistry and neuroanatomy that are associated with depressive disorders. Many of behavioral and neurobiological changes produced by repeated glucocorticoid administration in animals are reversed by antidepressant treatment (Ago et al., 2008; David et al., 2009).

Major depression is a highly recurrent disorder, as it is frequently characterized by repeated episodes over the life course. Some evidence estimates that 60% of individuals who have had one depressive episode will have another, 70% of depressed individuals with two episodes will have a third, and finally, 90% of depressed individuals with three episodes will

have a fourth episode (Hollon et al., 2006; Monroe & Harkness, 2005; Pettit, Lewinsohn, & Joiner, 2006). The probability of recurrence increases with the earlier age of onset of the initial episode, the severity of the episode, and with poor quality of recovery from the episode (Judd et al., 2000; Pettit et al., 2006). However, current animal models of depression fail to provide any means to understand the typical cycling of episode re-establishment across time that occurs in most depressed patients. Accordingly, in the current study, we aimed to recapitulate the cyclical disease course of depression in the animal model and test the hypothesis of sensitization of stress-induced depression-like behavior over two cycles of CORT treatment. This question was addressed by comparing effects of repeated and cyclic CORT treatment on depressive-like behaviors in rats.

2.2. Materials and Methods

2.2.1. Subjects

Male Long-Evans rats ($n = 30$) were purchased from Charles River Laboratories, Montreal, QC, Canada. At the time of arrival, the rats weighed approximately 200-250 grams. The rats were individually housed in standard polypropylene cages with Purina rat chow and water available ad libitum. The colony room was maintained at a temperature of 21°C with a 12 h :12 h light/dark cycle (lights on at 7:00 a.m.). All experimental procedures were conducted during the light phase of the light/dark cycle and in accordance with the Canadian Council on Animal Care and the University of Saskatchewan Committee on Animal Care.

2.2.2. Treatment with CORT

The rats were handled for seven days in the colony room prior to any experimental manipulations. At the end of handling, the rats were weight matched and assigned to one of

three different treatment groups: 20 mg/kg CORT (n = 10), 40 mg/kg CORT (n = 10), or vehicle (n = 10). CORT or vehicle was delivered once per day via subcutaneous injection between 09:00 and 11:00 h. CORT (Steraloids, Inc., Newport, RI) was suspended in 0.9 (w/v) physiological saline with 2% (v/v) polyoxyethylene glycol sorbitan monooleate (Tween-80, Sigma-Aldrich, St. Louis, MO). Each cycle of injections comprised 21 consecutive days, followed by 21 days of injection-free recovery. During recovery periods the rats were left untouched in their home cages. The experimental design is depicted in Figure 2-1.

2.2.3. Body Weight

All rats were weighed on a daily basis during CORT treatment periods and once at the end of each recovery period. Body weights were recorded for later analyses.

2.2.4. Behavioral Testing

A modified one-day version of FST was used to assess depression-like behavior, as described previously (Gregus et al., 2005). No pre-test session was conducted; each rat was tested in the swim tank only once to evaluate “helplessness” induced by prior exposure to CORT injections. The modified one-day version of a FST was chosen because previous research in our laboratory has shown comparable behavioral results with both a one-day and a two-day version of the FST. Furthermore, a one-day FST protocol seems to be appropriate to assess depression-like behavior in experiments with prior exposures to stress by eliminating potential confounding effects of memory from the first day of FST exposure on behavior during the second “testing” day (Marks et al., 2009).

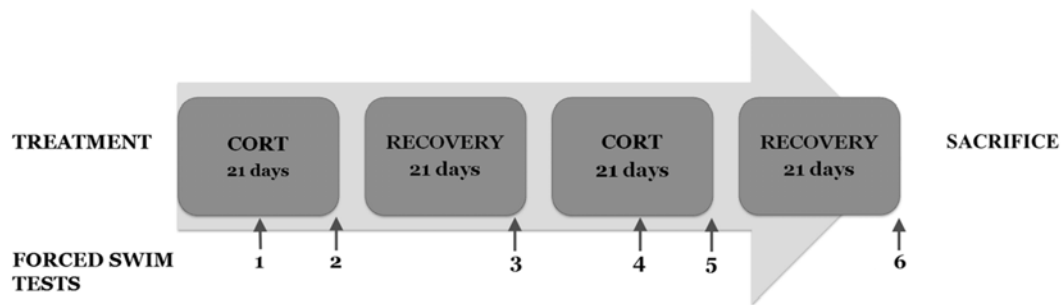


Figure 2-1. A schematic of the experimental design. Two groups of corticosterone (CORT)-treated rats and one group of vehicle-treated rats were exposed to two cycles of treatments and recovery periods. Behavioral testing was repeatedly administered in the middle and in the end of each treatment, and in the end of each recovery period.

The FST was conducted in a rectangular Plexiglas swim tank (25 cm long×25 cm wide×60 cm high), filled with 27±2 °C water to a depth of 30 cm. Rats were transported from the colony room into an adjacent behavioral testing room. Each rat was placed individually into swim tank for 10 minutes. Behavior in the swim tank was video recorded with a camera mounted on a tripod horizontal to the swim tank. After 10 min, the rat was removed from the tank, towel dried and placed back in its home cage to dry under a heat lamp for approximately 20 min. The water in the swim tank was changed in between rats. Time spent immobile, swimming and climbing, as well as the latency to become immobile in the FST has been assessed as measures of depression-like behavior.

The rats were repeatedly tested during the course of the experiment. The FSTs were conducted in the middle (day 10) and at the end (day 22) of each period of CORT treatment, and at the end of each recovery period (Figure 2-1). The within-subject research paradigm was chosen because it was used in a previous study in which naïve rats treated with antidepressants showed a significant decrease in immobility rates over several repetitive FSTs, indicating a decrease in depression-like behavior with each consecutive behavioral test (Mezadri, Batista, Portes, Marino-Neto, & Lino-de-Oliveira, 2011).

2.2.5. Statistical Analyses

The data were analyzed using the statistical package for social sciences (SPSS) software program (v. 18). Repeated measures analysis of variance (ANOVA) was conducted to measure main effects and interactions, with treatment as the between-subjects factor and time as the within-subjects factor. The significance level was set at $p = 0.05$ for all statistical comparisons. In addition, separate one-way ANOVA's were used to compare the effect of treatment on dependent variables at each experimental time point. This was followed by the Fisher's least significant difference (LSD) post hoc t-tests when appropriate.

2.3. Results

2.3.1. Body Weight

Mauchly's test indicated that the assumption of sphericity was violated, $X^2(14) = 102.02, p = 0.001$, therefore the degrees of freedom were corrected using Greenhouse-Geisser sphericity estimates. One-way repeated measures ANOVA revealed a significant main effect of time on body weight [$F(1.9, 49.9) = 350.7, p = 0.001$], as well as a significant interaction of treatment by time [$F(3.8, 49.9) = 4.042, p = 0.007$]. There was no significant main effect of treatment on body weight ($p = 0.156$).

Group differences at each time point of the experiment were investigated using planned comparisons of separate one-way ANOVAs. Figure 2-2 shows the mean body weights for the rats in each group during the two-cycles of CORT treatment. In the first cycle, the CORT rats gained dramatically less weight than did the vehicle rats. The statistical analysis demonstrated a significant main effect of group on injection day 10, [$F(2,27) = 5.405, p = 0.011$], and injection day 21, [$F(2,27) = 3.266, p = 0.05$]. Post hoc tests (LSD) revealed that the rats treated with 40 mg/kg CORT weighed significantly less than the vehicle rats after ten days of injections ($p = 0.003$), and continued to do so until day 21 of CORT treatment ($p = 0.018$). No significant differences in body weight among all groups of rats were detected after the recovery in cycle 1 ($p = 0.941$).

In the second cycle, the rats treated with CORT weighed less than the vehicle rats by injection day 10, but the difference between groups missed statistical significance ($p = 0.098$). However, the differences between the groups in body weight were significant by day 21 [$F(2,27) = 4.845, p = 0.016$]. Post hoc (LSD) analyses revealed that the 40 mg/kg CORT group was significantly different from the vehicle group on day 21 ($p = 0.004$). After the final recovery period, no differences in body weight between groups were observed ($p = 0.419$).

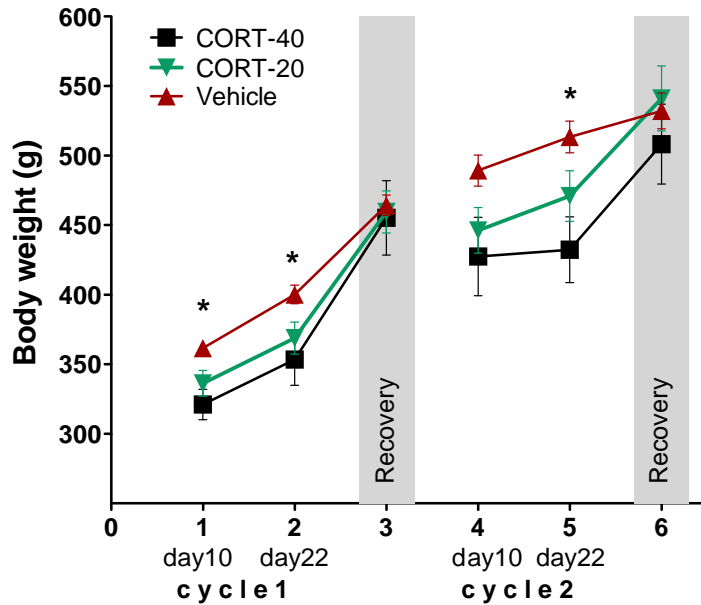


Figure 2-2. The effect of one and two cycles of repeated corticosterone (CORT) injections on rat body weight. Rats treated with CORT displayed decreased body weight throughout the two cycles of treatment, with the exception of recovery periods and early CORT re-exposure. Asterisks indicate significant differences between groups ($p < 0.05$) at specific time points. Error bars denote the standard error of the mean (SEM).

2.3.2. Forced Swim Tests

2.3.2.1. Immobility

Mauchly's test indicated that the assumption of sphericity was violated, $X^2(14) = 30.6$, $p = 0.006$, therefore the degrees of freedom were corrected using Greenhouse-Geisser sphericity estimates. One-way repeated measures ANOVA revealed a significant main effect of time on immobility [$F(3.7, 94.96) = 19.61$, $p = 0.001$], but no significant interaction of treatment by time ($p = 0.09$). There was also a significant main effect of treatment on immobility [$F(2, 26) = 3.28$, $p = 0.05$]. Post hoc (LSD) analyses confirmed that the difference occurred between the 40 mg/kg CORT group and the vehicle group ($p = 0.023$).

Group differences at each time point of the experiment were investigated using planned comparisons of separate one-way ANOVAs. The results of all FSTs conducted in the experiment are shown in Figure 2-3. CORT treatment had no significant effect on immobility behavior in the FST 1, after 10 days of injections ($p = 0.187$). There was a significant increase in time spent immobile in CORT-treated groups in FST-2, after 21 days of injections [$F(2,27) = 3.788$, $p = 0.035$]. The post hoc (LSD) analyses revealed that the difference lies between the 40 mg/kg CORT group and the vehicle group ($p = 0.024$), as well as between the 20 mg/kg CORT group and the vehicle group ($p = 0.025$). No difference ($p = 0.906$) between groups was found in FST-3, which occurred after recovery.

In the second cycle, CORT treatment had a significant effect on immobility time during both FST-4 [$F(2,27) = 6.597$, $p = 0.005$] and FST-5 [$F(2,27) = 6.272$, $p = 0.006$] testing, after 10 and 21 days of injections, respectively. The post hoc (LSD) tests confirmed that the 40 mg/kg CORT group showed more immobility than the vehicle group [$p = 0.004$ in FST-4 and $p = 0.004$ in FST-5], and that the 20 mg/kg group also showed more immobility than the vehicle group [$p = 0.005$, in FST-4 and $p = 0.006$, in FST 5]. There were no significant differences among the groups in immobility behavior after recovery ($p = 0.283$).

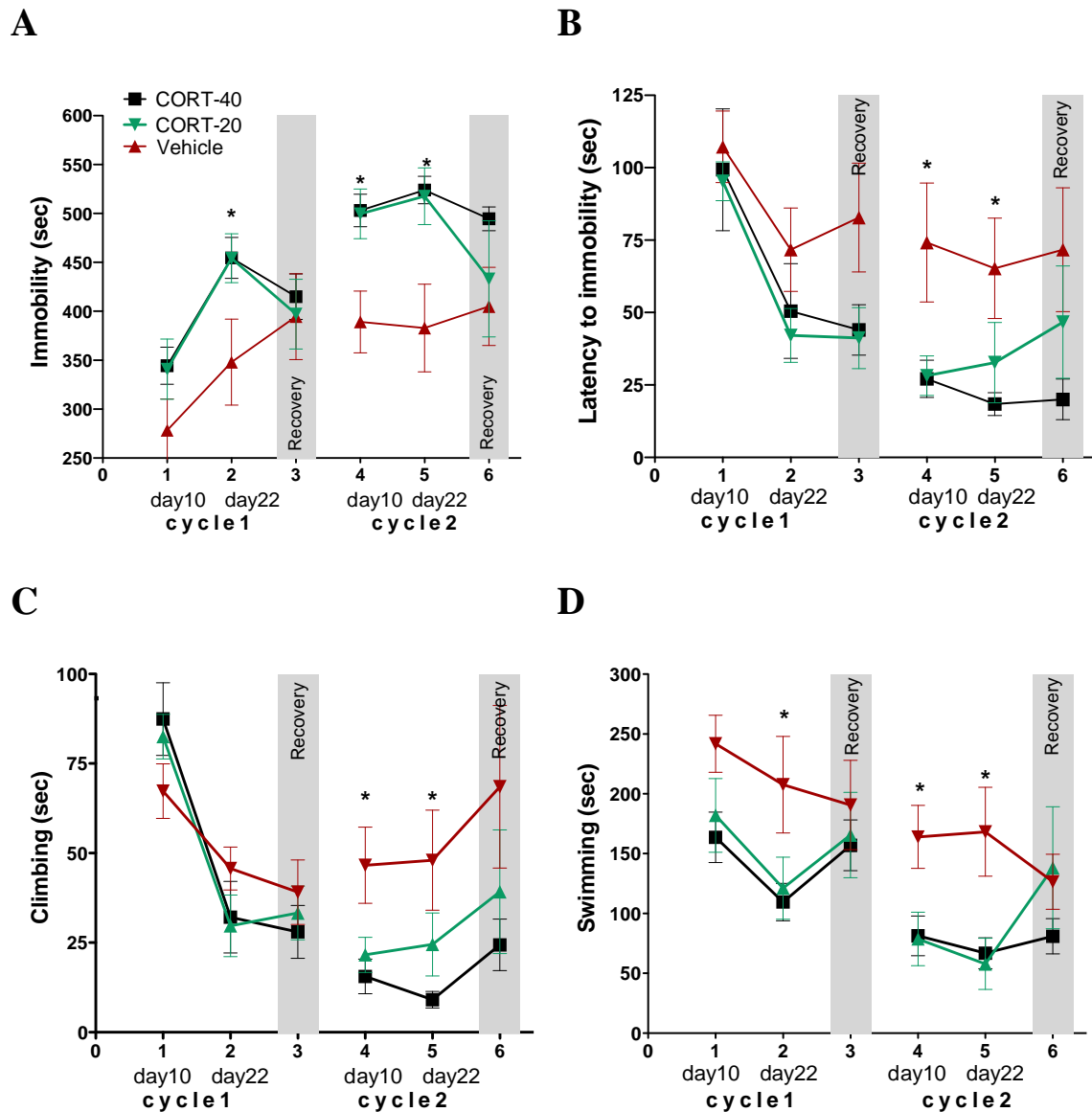


Figure 2-3. The effect of one and two cycles of repeated corticosterone (CORT) injections on behavior in the forced swim test. Panel (A) shows the mean time spent immobile, panel (B) - the mean latency to immobility, panel (C) - the mean time spent climbing, and panel (D) - the mean time spent swimming. Rats treated with CORT spent significantly more time immobile and less time swimming and climbing at the end of the 1st treatment cycle. Following CORT re-exposure in the second cycle, the CORT rats showed a sensitized increase in depression-like behavior after only ten days of treatment. The asterisk indicates a significant difference between groups at specific time points ($p < 0.05$). Error bars denote the standard error of the mean (SEM).

2.3.2.2. Latency to Immobility

Mauchly's test indicated that the assumption of sphericity was violated, $X^2(14) = 32.18, p = 0.004$, therefore the degrees of freedom were corrected using Greenhouse-Geisser sphericity estimates. Repeated measures ANOVA revealed a significant main effect of time on latency to immobility [$F(3.1, 74.6) = 13.03, p = 0.001$], but no significant interaction of treatment by time ($p = 0.31$). There was also a significant main effect of treatment on latency to immobility [$F(2, 24) = 3.28, p = 0.036$]. Post hoc (LSD) analyses confirmed significant differences between the 40 mg/kg CORT group and the vehicle group ($p = 0.018$), as well as between the 20 mg/kg CORT group and the vehicle group ($p = 0.03$).

Group differences at each time point of the experiment were investigated using planned comparisons of separate one-way ANOVAs. Figure 2-3B shows the mean latency to immobility in each group for the FSTs conducted during cycle 1 and cycle 2. In cycle 1, no significant group differences in latency to immobility were seen during any of the FSTs (all p 's > 0.05).

In cycle 2, there was a significant decrease in the latency to immobility in both groups of CORT-injected rats after ten days of treatment, [$F(2,27) = 4.261, p = 0.025$]. That is, both the 40 mg/kg CORT rats ($p = 0.016$) and the 20 mg/kg CORT rats ($p = 0.019$) had a shorter latency to immobility compared to the vehicle rats. A similar effect of CORT was seen during FST-5, after 21 days of treatment, there was a significant difference among the groups [$F(2,27) = 3.524, p = 0.045$], and post hoc (LSD) analyses revealed that the 40 mg/kg CORT group had a shorter latency to immobility than the vehicle group ($p = 0.015$). There were no significant group differences in latency to immobility during FST-6, after recovery ($p > 0.05$).

2.3.2.3. Climbing

Mauchly's test indicated that the assumption of sphericity was violated, $X^2(14) = 47.06, p = 0.001$, therefore the degrees of freedom were corrected using Greenhouse-Geisser sphericity estimates. One-way repeated measures ANOVA revealed a significant main effect of time on climbing [$F(3.07, 79.82) = 16.45, p = 0.001$], as well as a significant interaction of treatment by time [$F(6.14, 79.82) = 2.29, p = 0.042$]. There was no significant main effect of treatment on climbing ($p = 0.144$).

Group differences at each time point of the experiment were investigated using planned comparisons of separate one-way ANOVAs. There were no significant group differences in climbing behavior during any of the FSTs in cycle 1 (all p 's > 0.05). However, CORT had a significant effect on climbing behavior in cycle 2. In FST 4, there was a significant group difference in climbing after 10 days of treatment [$F(2,27) = 5.040, p = 0.014$], and post hoc (LSD) analyses revealed that the 40 mg/kg CORT rats ($p = 0.006$) and 20 mg/kg CORT rats ($p = 0.023$) both showed less climbing behavior than the vehicle rats. In FST-5, after 21 days of treatment, there was also a significant group difference in climbing [$F(2,27) = 4.330, p = 0.023$], and this time only the 40 mg/kg CORT rats showed less climbing than the vehicle rats ($p = 0.007$). There were no significant group differences in climbing on FST-6, after recovery ($p > 0.05$).

2.3.2.4. Swimming

Mauchly's test indicated that the assumption of sphericity was violated, $X^2(14) = 35.43, p = 0.001$, therefore the degrees of freedom were corrected using Greenhouse-Geisser sphericity estimates. Repeated measures ANOVA revealed a significant main effect of time on swimming behavior [$F(3.36, 87.43) = 11.17, p = 0.001$], but no significant interaction of treatment by time ($p = 0.22$). There was also a significant main effect of treatment on latency

to immobility [$F(2, 26) = 3.18, p = 0.05$]. Post hoc (LSD) analyses confirmed a significant difference between the 40 mg/kg CORT group and the vehicle group ($p = 0.024$).

Group differences at each time point of the experiment were analyzed using planned comparisons of separate one-way ANOVAs. CORT treatment had significant effects on swimming behavior during both cycles. In cycle 1, although there were no group differences in swimming in FST-1, the groups did differ on this measure in FST-2 [$F(2,27) = 3.385, p = 0.049$]. Post hoc (LSD) analyses showed that the 40 mg/kg CORT rats ($p = 0.025$) and the 20 mg/kg CORT rats ($p = 0.045$) both showed less swimming than did the vehicle rats. No significant group differences were found in FST-3, after recovery ($p > 0.05$).

In cycle 2, there was a significant main effect of treatment on time spent swimming in FST-4, after 10 days of treatment, [$F(2,27) = 4.782, p = 0.017$]. Post hoc (LSD) analyses revealed that both the 40 mg/kg CORT rats ($p = 0.014$) and the 20 mg/kg CORT rats ($p = 0.011$) swam less than the vehicle rats. The same pattern of results was seen in FST-5, after 21 days of treatment. That is, there was an overall main effect of treatment on swimming during this test [$F(2,27) = 6.272, p = 0.006$], and post hoc (LSD) analyses showed that both the 40 mg/kg CORT ($p = 0.01$) and 20 mg/kg CORT rats ($p = 0.005$) swam less than the vehicle rats. There were no group differences in swimming groups in FST-6, after recovery ($p > 0.05$).

2.3.3. FST Behaviors Controlled for Body Weight

To account for the influence of body weight increases on FST behaviors, ratio scores were created: $T/BW = \text{ratio score}$, where T is time the subject spent engaging in FST behaviors (immobility, climbing, etc.) in seconds, and BW is subject's body weight in grams at the time of testing. Figure 2-4 shows the mean ratio scores in each treatment group for the FSTs conducted during cycle 1 and cycle 2.

2.3.3.1. Immobility to Body Weight (IBW) Ratio

Mauchly's test indicated that the assumption of sphericity was violated, $X^2(14) = 31.82, p = 0.004$, therefore the degrees of freedom were corrected using Greenhouse-Geisser sphericity estimates. Repeated measures ANOVA revealed a significant main effect of time on immobility to body weight (IBW) ratios [$F(3.62, 94.086) = 13.55, p = 0.001$], as well as a significant interaction of treatment by time [$F(7.23, 94.086) = 3.14, p = 0.005$]. There was also a significant main effect of treatment on IBW ratios [$F(2, 26) = 7.32, p = 0.003$]. Post hoc (LSD) analyses confirmed significant differences between the 40 mg/kg CORT group and the vehicle group ($p = 0.001$), as well as between the 20 mg/kg CORT group and the vehicle group ($p = 0.027$).

Group differences at each time point of the experiment were analyzed using planned comparisons with separate one-way ANOVAs. CORT treatment had significant effects on the immobility/body weight (IBW) ratio during both cycles. In cycle 1, the treatment groups differed on this measure in both FST-1 [$F(2,27) = 4.44, p = 0.022$] and FST-2 [$F(2,27) = 6.84, p = 0.004$]. Post hoc (LSD) analyses revealed that the 40 mg/kg CORT rats ($p = 0.009$ and $p = 0.002$) and 20 mg/kg CORT rats ($p = 0.034$ and $p = 0.01$) both showed smaller ratio scores than did the vehicle rats in FST-1 and FST-2, respectively. No significant group differences were found after recovery on FST-3 ($p = 0.701$).

In cycle 2, there was a significant main effect of treatment on IBW ratios after 10 days of treatment on FST-4 [$F(2,27) = 11.27, p = 0.001$]. Post hoc (LSD) analyses revealed that both the 40 mg/kg CORT rats ($p = 0.001$) and the 20 mg/kg CORT rats ($p = 0.002$) had lower IBW ratio scores than the vehicle rats. The same pattern of results was seen in FST-5, after 21 days of treatment. That is, there was an overall main effect of treatment on IBW ratio scores during this test [$F(2,27) = 13.95, p = 0.001$], and post hoc (LSD) tests showed that

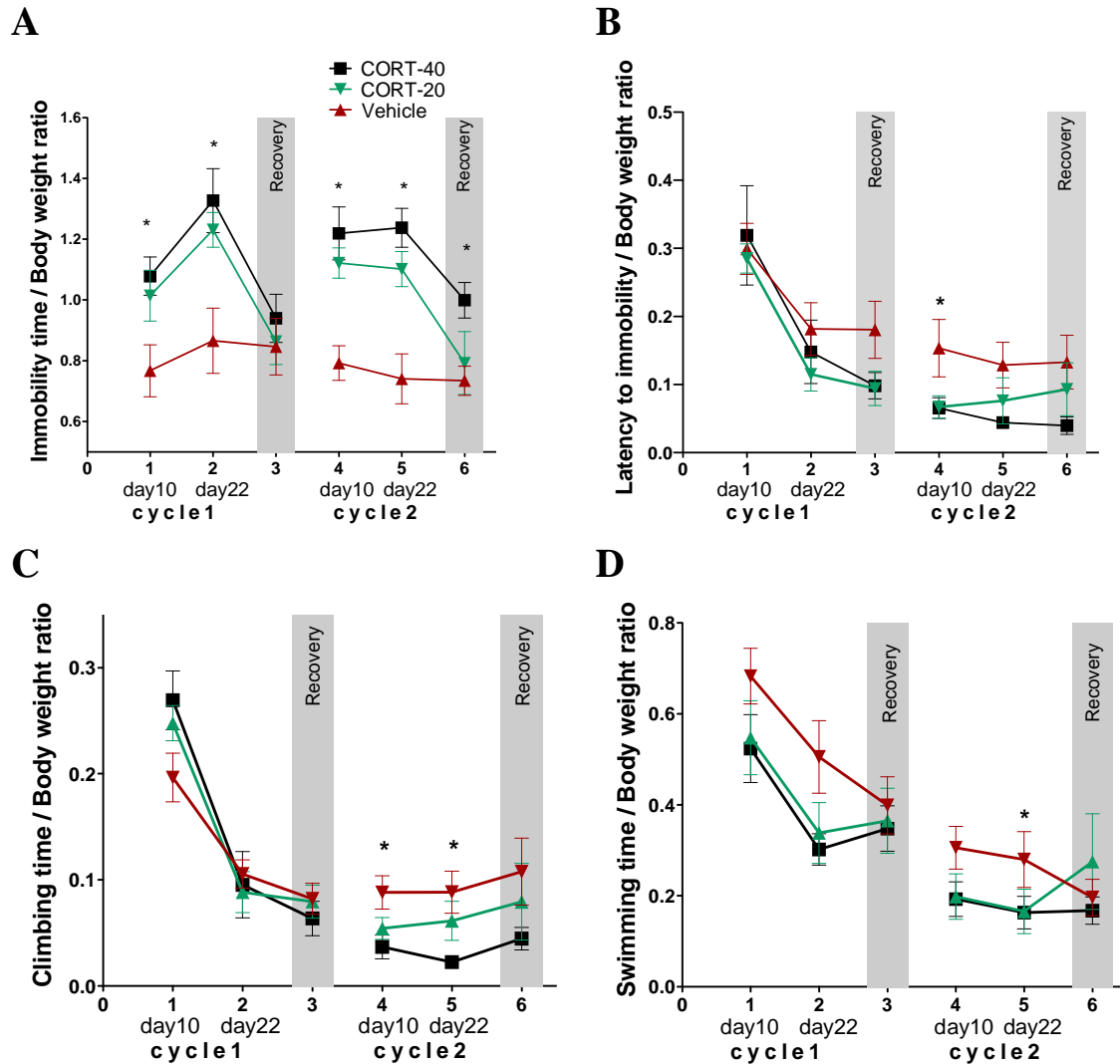


Figure 2-4. The effect of one and two cycles of repeated corticosterone (CORT) injections on the forced swim test (FST) behavior plotted by body weight. Panel (A) shows the immobility time to body weight ratio, panel (B) shows the latency to immobility to body weight (IBW) ratio, panel (C) shows the climbing time to body weight ratio, and panel (D) shows the time spent swimming to body weight ratio. Rats treated with CORT higher IBW ratio scores at the end of treatment cycle one. Following CORT re-exposure in the second cycle, the CORT rats showed a sensitized increase in IBW scores after only ten days of treatment. It was also noted that CORT rats showed worsened recovery of IBW scores at the end of the second recovery period. Asterisks indicate significant differences between groups at specific time points ($p < 0.05$). Error bars denote the standard error of the mean (SEM).

both the 40 mg/kg CORT ($p = 0.001$) and 20 mg/kg CORT rats ($p = 0.001$) had lower ratio scores than the vehicle rats. Additionally, a significant main effect of treatment on IBW ratios was found in FST-6, after recovery [$F(2,27) = 3.37, p = 0.05$]. The post hoc (LSD) analyses indicated that the difference occurred between the 40 mg/kg CORT and the vehicle rats ($p = 0.02$).

2.3.3.2. Latency to Immobility to Body Weight (LIBW) Ratio

Mauchly's test indicated that the assumption of sphericity was violated, $X^2(14) = 57.26, p = 0.001$, therefore the degrees of freedom were corrected using Greenhouse-Geisser sphericity estimates. Repeated measures ANOVA revealed a significant main effect of time on latency to immobility/body weight (LIBW) ratios [$F(2.5, 59.82) = 24.55, p = 0.001$]. There was no significant main effect of treatment on LIBW ratios ($p = 0.42$), and no significant interaction of treatment by time ($p = 0.116$).

Our *a priori* predictions led us to investigate group differences at each time point of the experiment using planned comparisons with separate one-way ANOVAs. In cycle 1, no significant group differences in LIBW ratios were seen during any of the FSTs (all p 's > 0.05). In cycle 2, there was a significant decrease in the LIWB ratio scores in both groups of CORT-injected rats after 10 days of treatment, [$F(2,27) = 3.34, p = 0.05$]. That is, both the 40 mg/kg CORT rats ($p = 0.032$) and the 20 mg/kg CORT rats ($p = 0.035$) had lower LIBW scores compared to the vehicle rats. There were no significant group differences in LIBW ratio scores during FST-5 and FST-6 ($p > 0.05$).

2.3.3.3. Climbing to Body Weight (CBW) Ratio

Mauchly's test indicated that the assumption of sphericity was violated, $X^2(14) = 42.9, p = 0.001$, therefore the degrees of freedom were corrected using Greenhouse-Geisser

sphericity estimates. Repeated measures ANOVA revealed a significant main effect of time on climbing/body weight (CBW) ratios [$F(3.43, 89.22) = 38.19, p = 0.001$], as well as a significant interaction of treatment by time [$F(6.86, 89.22) = 2.9, p = 0.009$]. There was no significant main effect of treatment on CBW ($p = 0.409$).

Group differences at each time point of the experiment were investigated using planned comparisons of separate one-way ANOVAs. In cycle 1, we found no significant group differences in CBW ratios during the FST1-3 (all p 's > 0.05). In cycle 2, there was a significant main effect of treatment on CWB ratio scores in FST-4 [$F(2,27) = 3.9, p = 0.032$] after 10 days of treatment, and in FST-5 after 21 days of treatment [$F(2,27) = 3.32, p = 0.05$]. That is, both the 40 mg/kg CORT rats ($p = 0.013$) and the 20 mg/kg CORT rats ($p = 0.05$) had lower CBW scores compared to the vehicle rats in FST-4. And only the 40 mg/kg CORT rats ($p = 0.013$) had lower CBW ratio scores than the vehicle group in FST-5. There was no significant group difference in CBW ratio scores during FST-6 after recovery ($p = 0.201$).

2.3.3.4. Swimming to Body Weight (SBW) Ratio

Mauchly's test indicated that the assumption of sphericity was violated, $X^2(14) = 40.33, p = 0.001$, therefore the degrees of freedom were corrected using Greenhouse-Geisser sphericity estimates. Repeated measures ANOVA revealed a significant main effect of time on swimming/body weight (SBW) ratios [$F(3.7, 84.99) = 25.82, p = 0.001$]. There was no significant main effect of treatment on SBW ratios ($p = 0.41$), and no significant interaction of treatment by time ($p = 0.187$).

Our *a priori* predictions led us to investigate group differences at each time point of the experiment using planned comparisons with separate one-way ANOVAs. In cycle 1, no significant group differences in SBW ratios were found during the FST1-3 (all p 's > 0.05). In cycle 2, no significant group differences in SBW ratios were found during the FST-4, after 10

days of injections ($p > 0.078$). There was a significant decrease in the SWB ratio scores in both groups of CORT-injected rats after 21 days of treatment [$F(2,27) = 3.61, p = 0.041$]. That is, both the 40 mg/kg CORT rats ($p = 0.043$) and the 20 mg/kg CORT rats ($p = 0.019$) had lower SBW scores compared to the vehicle rats. There were no significant group differences in SBW ratio scores during FST-6, after recovery ($p = 0.09$).

2.3.4. Changes in Recovery of Immobility Behavior Across Two Cycles of Treatment

To evaluate the changes in the quality of recovery across two cycles of CORT treatment, the percent change in immobility time and IBW ratio in the 20 mg/kg and 40 mg/kg CORT rats were calculated. For each rat, we calculated the difference in FST scores on day 22 and after recovery and then divided that amount by the day 22 FST score and multiplied by 100. We then averaged the obtained values for rats in each group to get a group mean.

The results are presented in Table 2-1. Repeated measures ANOVA revealed no significant main effect of time on percent change in immobility ($p = 0.72$) in either the CORT 20 and CORT 40 mg/kg groups. However, there was a significant main effect of time on percent change of IBW ratio scores [$F(1,17) = 13.06, p = 0.002$]. The post hoc (LSD) analyses confirmed that the difference occurred between percent change in cycle 1 and cycle 2 in the 40 mg/kg CORT rats ($p = 0.002$).

2.4. Discussion

The purpose of this experiment was to determine whether the effects of CORT on depression-like behavior can be sensitized with multiple cycles of exposure. Our results support this idea. There were no significant differences between the vehicle and CORT-

Table 2-1. Changes in recovery over two cycles of corticosterone (CORT) treatment. The values listed are the percent change in immobility time and immobility time corrected for body weight (IBW) ratio in the 20 mg/kg and 40 mg/kg CORT groups. Asterisks (*) represent statistically significant difference between the labeled values. For details, see the text.

	Immobility Time, % change		Immobility to Body Weight Ratio, % change	
	CORT- 20 mg/kg	CORT- 40 mg/kg	CORT- 20 mg/kg	CORT- 40 mg/kg
Recovery, cycle one	13.6 ± 5.5%	8.5 ± 5.2%	53.1 ± 44.7%	42.3 ± 16.9% *
Recovery, cycle two	19.9 ± 6.0%	5.1 ± 5.6%	29.7 ± 20.7%	19.2 ± 9.3% *

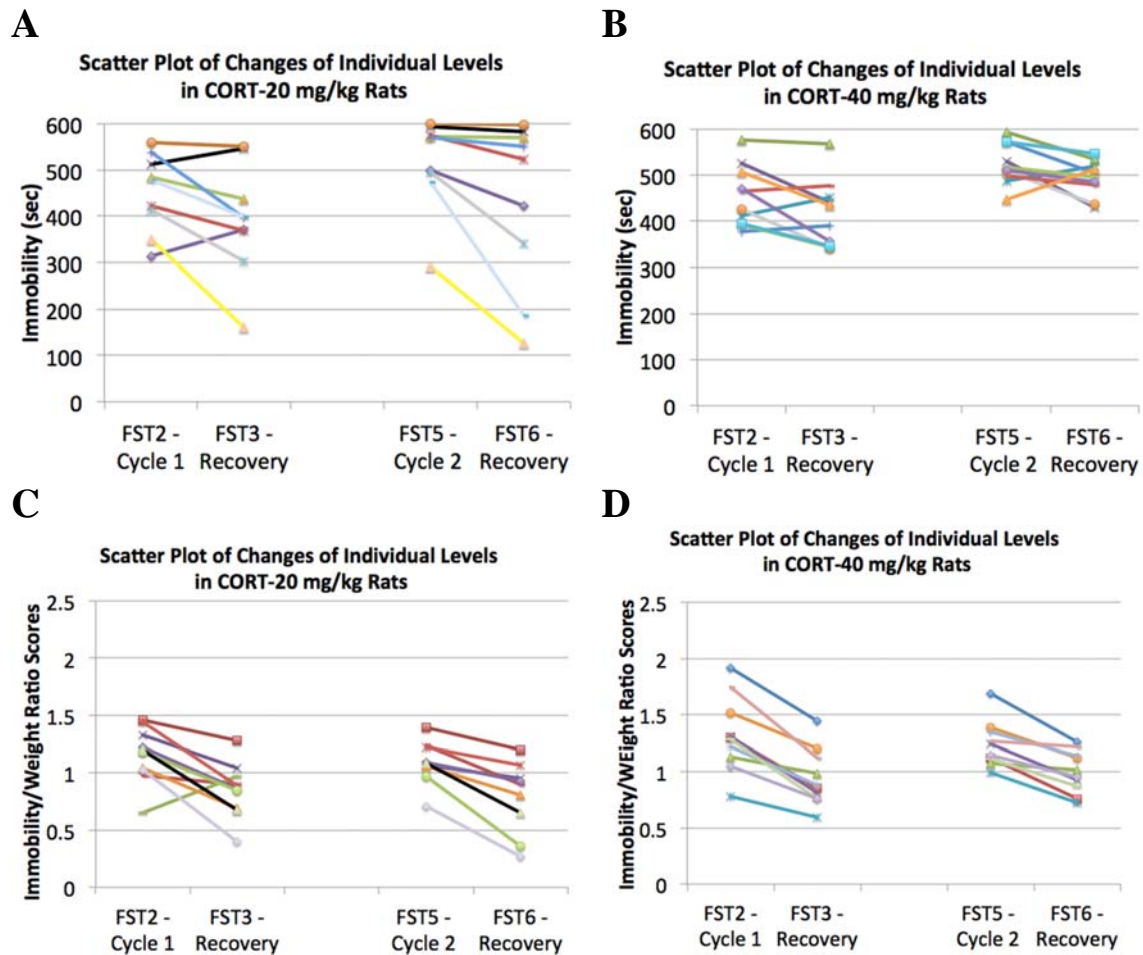


Figure 2-5. Scatter plots of changes in immobility and immobility to body weight (IBW) ratio scores in individual rats. Panels (A) and (C) show immobility time and IBW ratio scores in the 20 mg/kg corticosterone (CORT) rats; panels (B) and (D) and show immobility time and IBW ratio scores in the 40 mg/kg CORT rats. CORT rats exhibit high variability in the recovery rate of immobility time and IBW ratio scores during the first recovery period, and less individual variability during the second recovery period.

treated rats in FST behaviors (immobility, climbing, swimming, and latency to immobility onset) after only ten days of injections in cycle 1. As previously reported (Brotto, Gorzalka, & Barr, 2001; Mitra & Sapolsky, 2008), ten days of daily CORT injections are not sufficient to produce significant increases in depression-like behavior in rats. However, CORT did significantly alter immobility and swimming behavior after 21 days of injections. Rats treated with 40 mg/kg or 20 mg/kg CORT spent significantly more time immobile and less time swimming compared to control rats. This result supports previous findings from our laboratory (Johnson et al., 2006; Lussier et al., 2013; Marks et al., 2009) and others (Brummelte & Galea, 2010; Waters & McCormick, 2011; Workman, Brummelte, & Galea, 2013; Zhao, Ma, et al., 2008). Specifically, rats injected with 40 mg/kg CORT for 21 consecutive days reliably display an increase in immobility in the FST, which typically reflects a depressive phenotype in rodents (Cryan, Valentino, & Lucki, 2005).

A novel aspect of this experiment was providing rats with a recovery period and then administering CORT for one additional cycle. Our data revealed that 21 days of recovery was sufficient for both behavior and body weight to normalize, as seen in FST-3: CORT-treated rats showed a substantial decrease in time spent immobile and restored body weight gain to the levels of the control group. However, when the rats were re-exposed to CORT for the second time, we found greater immobility and reduced swimming and climbing in the FST. For example, we observed an early re-establishment of depression-like behavior after only ten days of CORT injections. Specifically, rats re-exposed to the second cycle of CORT spent significantly more time immobile and less time climbing and swimming compared to the vehicle-injected rats. This effect was seen with both doses of CORT. These findings suggest that only half of the initial duration of CORT treatment in the second cycle was sufficient to trigger significant increases in depression-like symptoms in rats that were pre-exposed to CORT treatment and allowed to recover.

Similar to our observations with immobility behavior, the latency to immobility measure also seemed to decrease over two cycles in CORT-treated rats. Specifically, there were no group differences in latency to immobility in cycle 1, but in cycle 2, both groups of CORT rats had a shorter latency to immobility on days 10 and 22 compared to the vehicle rats.

In addition to these behavioral changes, exposure to repeated CORT injections produced physiological alterations indicative of depression. Rats treated with 40 mg/kg and 20 mg/kg CORT had significant decreases in body weight gain in during both cycles of treatment. During the recovery periods, their body weight recuperated, and the CORT rats were no longer different from the vehicle rats. Several mechanisms described in the literature could explain the effect of CORT treatment on body weight loss. There is a curvilinear relationship between CORT and body weight: relatively low levels of CORT result in weight gain, whereas chronically high levels of CORT (or glucocorticoid receptor agonists) lead to body weight loss (Devenport, Knehans, Sundstrom, & Thomas, 1989; Santana et al., 1995). Chronic glucocorticoid exposure increases hepatic gluconeogenesis and glycogenolysis, antagonizes insulin utilization in skeletal muscles and white adipose tissue, and modulates insulin and glucagon secretion from pancreatic β and α cells. Collectively, these effects result in insulin resistance, hyperglycemia, and consequent losses of urinary glucose (loss of kilocalories) (Andrews & Walker, 1999; Di Dalmazi, Pagotto, Pasquali, & Vicennati, 2012; Kuo, Harris, & Wang, 2013). Additionally, glucose concentrated urine leads to polyuria (increases in urination frequency) and may result in dehydration and rapid weight loss (Harris, 2015). Finally, chronic and high levels of CORT inhibit protein synthesis promoting the breakdown of muscles, and increase lipolysis, eventually leading to a reduction in adipose tissue, muscle atrophy, and body weight loss (Tempel & Leibowitz, 1994; Tomas, Munro, & Young, 1979). However, in human depression, both body weight gain (atypical subtype) and

body weight loss (melancholic subtype) have been observed (American Psychiatric Association, 2013), which can be explained by different mechanisms, such as increased appetite, emotional eating, or, in contrast, reduced food intake due to anhedonia or socioeconomic factors (Konttinen, Männistö, Sarlio-Lähteenkorva, Silventoinen, & Haukkala, 2010; van Strien, Konttinen, Homberg, Engels, & Winkens, 2016; van Strien, van der Zwaluw, & Engels, 2010).

Several biological factors, such as strain, body weight, age, and sex, are known to influence rodent behavior in various behavioral tests, including the FST (Bogdanova, Kanekar, D'anci, & Renshaw, 2013). The mechanisms by which these factors affect rodent performance in behavioral tests are not well understood. Aging significantly impacts multiple body systems. Changes in the peripheral nervous system (neuromuscular junction) are known to cause a decline in motor control in older humans (Faulkner, Larkin, Claflin, & Brooks, 2007). Additionally, central brain changes (atrophy of the motor cortex and corpus callosum, degeneration of dopaminergic neurotransmitter system) may contribute to age-related motor declines (Carlsson & Winblad, 1976; Inoue et al., 2001; van Dyck et al., 2002). However, not all age-related changes in behavior can be explained by reduced motor ability. For example, Turner et al. (2012) demonstrated that older rats (18-20 months old) showed reduced exploration of the open arms in the elevated plus maze and greater immobility time in the FST compared with young adult rats (3-4 months old), whereas on the Rotarod and total locomotor activity assessment, all groups behaved similarly (Turner et al., 2012). There are striking changes in HPA axis function associated with older age, such as dramatic increases in basal cortisol levels (Van Cauter, Leproult, & Kupfer, 1996), diminished circadian rhythm amplitude of cortisol fluctuations (Ferrari et al., 2001; Van Cauter et al., 1996), and dysregulated HPA negative feedback (Otte et al., 2005). It is important to note that these

changes in the HPA function resemble those seen in depressed individuals irrespective of age (Gobinath, Mahmoud, & Galea, 2015).

The longitudinal design of the present experiment has allowed for potential confounding effects of age-related body weight gain on FST behavior. Therefore, we attempted to account for these effects by creating and analyzing ratio scores, where each FST behavior was converted to a ratio according to each rat's weight at the time of testing. Interestingly, the IBW ratio of the vehicle rats eliminated the nonspecific increase in immobility in these rats that was seen over the course of two cycles of testing. In contrast, the calculation of IBW ratios for both 40 mg/kg and 20 mg/kg CORT rats had no effect on our expected changes in immobility with 10 and 21 days of CORT treatment, and our expected decreases in immobility after CORT-free recovery periods.

Finally, our data provide some evidence to suggest a progressive worsening of recovery in CORT-treated rats, which is dependent on the dose of CORT being used. Specifically, the 40 mg/kg CORT rats showed a 8.5% reduction of immobility time and a 42.3% reduction in IBW after the recovery period in cycle 1. However, after the recovery period in cycle 2, this group showed only a 5.9% reduction in immobility time and a 19.2% reduction of IBW ratio (Table 2-1, Figure 2-5). Similarly, but to a smaller degree, the 20 mg/kg CORT rats showed a 13.6% reduction of immobility time and a 53.1% IBW ratio reduction after recovery in cycle 1, and a 19.9% reduction in immobility time and 29.7% IBW ratio reduction after recovery in cycle 2 (Figures 2-3A, 2-4A, 2-5, and Table 2-1). In the clinical literature, this phenomenon is described as “partial remission” or remission with residual symptoms (Fava, Ruini, & Belaise, 2007). Several clinical studies showed that more than 80% of responders to antidepressant medication displayed residual symptoms (Gastó, Navarro, Catalán, Portella, & Marcos, 2003; Nierenberg et al., 1999). Also, residual symptoms were found in depressed patients treated with psychotherapy (Karp et al., 2004;

Ogrodniczuk, Piper, & Joyce, 2004; Simons, Murphy, Levine, & Wetzel, 1986; Thase et al., 1992). Moreover, 30-88% of patients who were successfully treated with antidepressants and categorized as “remitted”, also had residual symptoms of depression (Paykel, 1985; Paykel et al., 1995). Importantly, the residual symptoms were repeatedly reported as strong predictors of subsequent relapses into depression, and therefore are considered to be an important problem in therapeutic interventions (Fava & Visani, 2008; Paykel, 1998; Petersen et al., 2004). Along with increased depression severity over time (Kessing, 2008), smaller inter-episode duration, and increased number of episodes (Kendler et al., 2001; Kessing, 1998), partial remission could be a major contributor to the progression of depression (i.e., progressive structural and functional brain changes produced by recurring depressive episodes) (Moylan, Maes, Wray, & Berk, 2012).

The newly presented model mimics the periodic change of behavior into and out of depression that is observed in patients with depressive disorders (Belmaker & Agam, 2008). Episode recurrence has been shown as one of the hallmarks of major depression disease course (Kennedy, Abbott, & Paykel, 2003; Monroe & Harkness, 2005). Furthermore, increasing evidence in human research (Liu & Alloy, 2010) supports the role of stress generation (sequence of dependent stress) in depression pathogenesis. Our results provide evidence for the kindling hypothesis of depression, which suggests that due to lasting neurobiological changes that occur with each bout of depression, progressively less stress is required to trigger subsequent depression episodes (Monroe & Harkness, 2005; Post, 1992).

The research focused on the development of novel antidepressants has had limited success, partly due to the lack of adequate animal models of depression. More than half of patients that recover from major depressive episodes experience a relapse (Hollon et al., 2006; Pettit et al., 2006). The absence of treatments that prevent the recurrence of depression can be explained, in part, by the absence of animal models showing spontaneous episodes of

depression. The development of new animal models with recurrent depressive-like episodes is, therefore, essential in this regard. Our model provides stronger construct and face validity compared with other existing animal models of depression. This animal model imitates the cyclical disease course of major depression over and above its ability to produce depressogenic effects on behavior (inhibited grooming and sexual behavior, increased helplessness), as well as physiological changes indicative of depression (weight loss, dysregulated HPA axis function) and finally, neurobiological alterations associated with depression disorders, such as hippocampal dendritic atrophy and decreased neurogenesis (Sterner & Kalynchuk, 2010).

Some of the limitations of this model of recurrent depression are potential confounding effects of repetitive FST exposure, aging, and body weight changes in the rats. Therefore, the interpretation of sensitization of depression-like behavior in the FST should be made with caution. One improvement that will be addressed in my next experiment is having separate groups of rats for each time point of FST, thus eliminating any repeated testing of the same rat.

CHAPTER 3

Cyclical Administration of CORT in an Animal Model of Recurrent Depression Results in Aggravation of Depressive-like Behaviors and Accompanying Changes in Neurobiological Markers

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Abstract

Exposure to chronic stress (or glucocorticoids) is a significant risk factor for depressive disorders. However, the mechanism by which stress might lead to depression is still unknown. Several hypotheses have suggested one such mechanism could involve stress-induced decreases in adult neurogenesis. The purpose of this experiment was to determine the effects of repeated and cyclic CORT administration on depression-like behavior, hippocampal neurogenesis, and its transcriptional regulation. Rats were given CORT injections or vehicle injections once per day for one, two, or three cycles (21 days each), followed by recovery periods. Rat behavior was measured in the forced swim, sucrose preference and open field tests to assess depressive symptoms and general locomotion at specific time points after CORT treatment or recovery. After behavioral testing, the rats were sacrificed for immunohistochemical analyses of adult hippocampal neurogenesis (immature neuron number, dendritic complexity), and the repressor element-1 silencing transcription factor (REST) expression in hippocampus. Our results demonstrate that repeated and cyclic exposure to high levels of CORT results in greater increases in depression-like behaviors, including higher immobility and lower swimming in the FST, and lower sucrose preference scores, but not in general locomotor behaviors. These changes in depression-like behaviors are paralleled by accumulated decreases in neurogenesis, specifically the number and dendritic complexity of doublecortin positive cells in dentate gyrus. Furthermore, we found a precise and time-dependent bi-directional regulation of REST levels within the hippocampus, which correlated with changes in depression-like behavior.

3.1. Introduction

Depression has an increasing prevalence worldwide, affecting over 350 million people in both developed and developing countries. The World Health Organization estimates that depression will be the leading cause of global disease burden by 2030 (World Health Organization, 2012).

In seeking a better understanding of the neurobiological mechanisms of depression, much of the current research has suggested that hippocampal dysfunction may be contributing to the etiology and treatment of this disease (Nestler, Gould, et al., 2002; Nestler, Barrot, et al., 2002). Numerous neuroimaging and post-mortem studies have reported hippocampal volume loss and abnormal cell morphology in depressed patients (MacQueen et al., 2003; Sheline, Wang, Gado, Csernansky, & Vannier, 1996; Sheline, Sanghavi, Mintun, & Gado, 1999; Sheline, Gado, & Kraemer, 2003). Aberrant adult hippocampal neurogenesis is hypothesized to be one of the reasons for the observed hippocampal pathology in major depression (Fournier & Duman, 2012; Sahay & Hen, 2007). Interestingly, preclinical studies of depression have demonstrated that various forms of chronic stress, including glucocorticoid administration, reduce the proliferation, survival, and differentiation of newborn hippocampal neurons in adult animals (Bambico & Belzung, 2013; Banasr & Duman, 2007). In addition, both clinical and preclinical data show that nearly all forms of antidepressant treatment, as well as exercise, enriched environments and other manipulations that lead to antidepressant-like behavioral effects promote adult neurogenesis (Boldrini et al., 2009; Fournier & Duman, 2012).

Adult neurogenesis is continuously controlled by multiple signalling pathways (Ables et al., 2010; Lie et al., 2005; Mira et al., 2010; Beckervordersandforth et al., 2015) and neurotransmitter systems (Deisseroth et al., 2004; Ge et al., 2006; Tozuka, Fukuda, Namba, Seki, & Hisatsune, 2005). Recent work has identified zinc finger protein REST (RE1-

silencing transcription factor, also known as NRSF) as a master factor for both differentiating precursor cells and adult neurons as well (Baldelli & Meldolesi, 2015). Initially, REST was known as a repressor of neuronal genes during embryonic development that was down-regulated once terminal neuronal differentiation has occurred (Ballas et al., 2005; Chong et al., 1995; Schoenherr & Anderson, 1995). However, growing evidence indicates that REST induction in mature neurons is subject to plasticity, for example, adult hippocampal granule cells and pyramidal neurons express high levels of REST (Calderone et al., 2003; Kuwabara et al., 2004; Palm et al., 1998; Sun et al., 2005), and adult neurons upregulate REST and specific target genes in response to ischemia or seizures (Calderone et al., 2003; Jessberger et al., 2007; Palm et al., 1998). An increase in REST was reported in normal aging in human cortical and hippocampal neurons, but a loss of hippocampal REST was found in patients with Alzheimer's disease, dementia and mild cognitive impairment (Lu et al., 2014). To my knowledge, the effects of repeated stress and depression on REST levels on the brain are largely unknown.

A longer course of depression is associated with more pronounced neurobiological changes, including atrophy of hippocampal volume (Bremner et al., 2000; Sheline et al., 1999; Sheline et al., 2003). Additionally, research in our laboratory shows that CORT-treated rats demonstrate gradual increases in depression-like behavior with 7, 14 and 21 days of treatment. These behavioral effects were accompanied by similarly gradual decreases in reelin expression in the dentate SGZ and decreases in the number and maturation rate of immature neurons in dentate gyrus (Lussier et al., 2013). These observations suggest that depression may be a progressive disease, with increasingly larger neurobiological perturbations leading to progressively severe symptoms and episode relapse. However, little is known about how the hippocampus is affected after multiple stress-induced depression-like episodes.

The specific aims of this experiment were: (1) to examine the effect of three cycles of CORT treatment with recovery between cycles on depression-like behavior, (2) to determine whether the progressive development of depression-like behavior in CORT-treated rats is paralleled by decreases in immature granule cell number and maturation, and (3) to examine changes in hippocampal REST protein immunoreactivity after one and three cycles of CORT treatment. Our results indicate that CORT does indeed produce greater effects on rat depression-like behavior (including higher immobility and less swimming in the FST, and lower sucrose preference scores) in cycles two and three compared to cycle one. These behavioral changes were paralleled by accumulated decreases in dentate gyrus immature neuron number and cell complexity. In addition, hippocampal REST levels displayed bi-directional changes depending on the number of CORT exposures.

3.2. Materials and Methods

3.2.1. Subjects

We used 183 male Long-Evans rats in this study (Charles River Laboratories, Canada). The rats weighed approximately 225-250 grams upon arrival to our facility. They were individually housed in standard polypropylene cages with water and food available ad libitum (unless otherwise indicated). The temperature of the colony room was maintained at 21°C and all experimental procedures were conducted during the light phase of the light/dark cycle (lights on at 8 a.m.). All experimental procedures were carried out according to a protocol approved by the University of Saskatchewan Committee on Animal Care and Supply.

3.2.2. Experimental Procedures

The rats were handled once per day for 7 days before the experimental procedures

were started. The rats were weight-matched and randomly assigned to one of the two treatment groups: repeated and cyclic CORT injections: 20 mg/kg, CORT (N = 91) or vehicle injections (N = 92). Both CORT and vehicle groups were divided into subgroups so that a different group of rats was evaluated at each time point: (1) 10 days of cycle one, (2) 21 days of cycle one, (3) recovery of cycle one, (4) 10 days of cycle two, (5) 21 days of cycle two, (6) recovery of cycle two, (7) 10 days of cycle three, (8) 21 days of cycle three, and (9) recovery of cycle 3. Each subgroup consisted of 20-22 rats (CORT and vehicle). These subgroups were employed to avoid the use of repeated exposure to the FST.

All injections were administered once per day for three cycles. Each cycle comprised 21 consecutive days of injections, followed by a 21-day injection-free recovery period (Figure 3-1). The injections were administered subcutaneously, between 9 and 11 a.m. CORT (Steraloids) was suspended in 0.9% (w/v) physiological saline with 2% (v/v) Tween-80 (Sigma Aldrich) and given in a volume of 1 ml/kg. The body weight of rats was recorded on each day of CORT treatment, and in the end of each recovery period.

3.2.3. Behavioral Testing

The behavioral testing was carried out in a different procedure room from the one used for the injections. We used the FST to assess depression-like behavior, the sucrose preference test (SPT) to assess anhedonia, and the open field test (OFT) to assess general locomotor behavior. All behavioral tests were administered in the middle (day 11) and in the end (day 22) of each cycle of CORT administration, and in the end of each recovery period.

As mentioned above, we used rats that were naïve to behavioral testing at each time point (Figure 3-1).

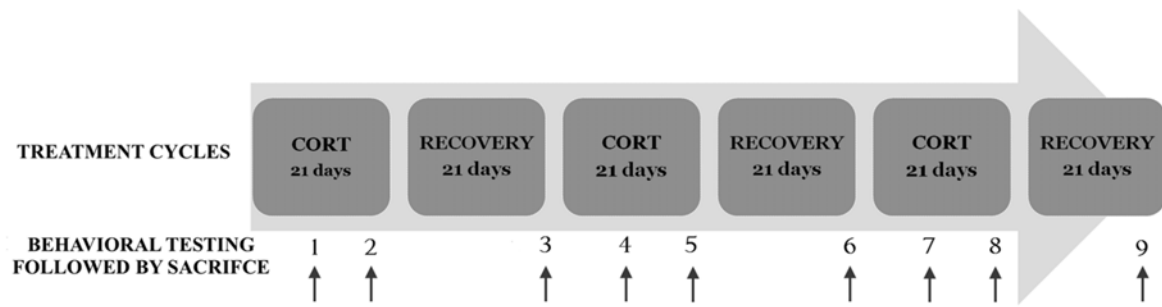


Figure 3-1. A schematic of the experimental design. Separate groups of corticosterone (CORT)- and vehicle-treated rats were exposed to varying durations of treatment and recovery. Behavioral testing was administered as shown in the diagram. At each time point, behavioral testing was followed by perfusions and brain tissue collection the next day.

3.2.3.1. Forced Swim Test

A modified version of the FST test protocol was used (Gregus et al., 2005). We chose to use a modified one-day version of the FST because previous research in our laboratory has shown comparable behavioral results with both a one-day and two-day versions of this test. Furthermore, a one-day FST protocol is appropriate to measure depression-like behavior in experiments with prior exposure to a repeated stress because it removes the possible confounding effects of memory from the first day of FST exposure on behavior during the second “testing” day (Marks et al., 2009). Briefly, rats were individually placed into a Plexiglas tank (25 cm W x 25 cm L x 60 cm H, 30 cm D) with 27 ± 2 °C water, for a duration of 10 minutes. The standard FST behaviors were scored: time spent immobile, time spent swimming, time spent climbing, and the latency to the first bout of immobility. Depression-like behavior was inferred from increases in time spent immobile and decreases in active FST behaviors (Gregus et al., 2005; Porsolt et al., 1978).

3.2.3.2. Sucrose Preference Test

Prior to any experimental procedures, all rats were given a 48-hour habituation to a two-bottle SPT procedure. During this time, the rats were presented with two 100 ml plastic bottles with 1% sucrose solution and allowed to drink freely. After this habituation, the rats were given their regular water until the SPT was conducted. The SPTs were conducted on the days following the FSTs. During the testing, the rats were provided with one bottle filled with 1% sucrose solution and another bottle filled with regular distilled water. The bottles were available for approximately 15 hours (overnight). The following morning the sucrose and water intake was measured and recorded.

3.2.3.3. Open Field Test

The OFTs were conducted following the SPTs. The open field apparatus (70 cm W × 70 cm L × 60 cm H) was a black wooden box with a transparent Plexiglas bottom and no top. The floor of the apparatus was divided into 36 equal squares by tape attached underneath the floor. Rats were individually placed in the corner of the open field and allowed to explore for five minutes. A video camera mounted on a tripod above the open field recorded the rats' activity. Total OFT activity, which included the number of lines crossed and the number of rears during the 5-minute session, was recorded. A rat was credited with one line cross when the center of rat's back moved over from one square and into the next square of the open field. A rat was credited with one rear each time it was standing on its rear limbs (Marks et al., 2009).

3.2.4. Perfusions and Tissue Preparation

After behavioral testing at each experimental time point, the rats were anesthetized with an overdose of intraperitoneal sodium pentobarbital and perfused transcardially with 0.1 M phosphate buffer (PB, pH 7.4) followed by 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed and postfixed in 4% (w/v) paraformaldehyde for 48 h at 4°C. Tissue was then sectioned at 50 µm using a vibratome (Vibratome 3000, Vibratome Company, St. Louis, MO, USA) and stored at -20°C in a cryoprotectant solution containing 30% (v/v) ethylene glycol, 1% (w/v) polyvinylpyrrolidone, and 30% (w/v) sucrose in 0.1 M PBS (pH = 7.4).

3.2.5. Immunohistochemical Procedures and Analyses

A subset of rats from each group (n = 4-5) was randomly selected for immunohistochemical analyses. Every 12th section of the hippocampus (from approximately -2.40 mm to -3.94 mm from bregma) was used for immunostaining. All immunostaining

procedures were done in 6-well tissue culture plates. For each immunohistochemical preparation, hippocampal slices from each treatment group were counterbalanced across all 6-well culture plates to ensure consistency in the immunohistochemical processing. Finally, to confirm the specificity of antibodies used in immunostaining, one well with brain sections served as a control and was subjected to immunohistochemical procedures in the absence of the primary antibody. We did not observe any immunoreactivity in the absence of the primary antibodies.

3.2.5.1. Doublecortin (DCX)

To visualize immature neurons, DCX immunohistochemistry was done according to our previously published methods (Fournier et al., 2010). Briefly, free-floating tissue sections were rinsed in 0.1 M Tris-buffered saline (TBS, pH 7.4). Following rinsing, the sections were subjected to a heat-induced antigen retrieval in a sodium citrate buffer (pH 6.0) at 95 °C for 30 min. Sections were then blocked with 5% (v/v) normal goat serum (NGS), 1% (w/v) BSA, and 0.5% (v/v) Triton X-100 in 0.1 M TBS, and incubated with a rabbit anti-DCX monoclonal primary antibody (1:1000; Cell Signaling Technology, MA, USA); diluted in block solution) for 48 hours at 4°C. Then, the tissue was incubated in 5% (v/v) H₂O₂ for 30 min to block endogenous peroxidase activity, followed by incubation with a biotinylated secondary antibody (goat anti-rabbit; 1:500; Vector Laboratories, USA; diluted in 5% NHS and TBSx) for 2 hours at RT, then ABC for 1 hour (1:500, Vector Laboratories, USA). Immunolabelling was visualized with 0.025% (w/v) 30-diaminobenzidine (DAB, Sigma Aldrich, St. Louis, MO), 4.167% (w/v) nickel ammonium sulphate, and 0.002% (v/v) H₂O₂ diluted in 0.175M sodium acetate. After approximately 5 minutes, the sections were rinsed in 0.175 M sodium acetate to stop the DAB reaction. The sections were then mounted onto glass

slides, dehydrated, and coverslipped using Entellan resin (Millipore, USA) for light microscopic examination.

DCX cell counts and maturation analysis. We assessed the total number of DCX positive cells using the unbiased optical fractionator method. Five tissue sections per rat were quantified. We visualized the brain sections at 400X magnification on a Nikon E800 microscope with a motorized stage and a stereology system software (Stereo Investigator, MicroBrightField). DCX positive cells were counted in the SGZ and GCL in the dentate gyrus (from approximately -2.40 mm to -3.94 mm from bregma). Counting parameters included a dissector height of 15 μm , a sampling area of 90x90 μm , and a counting frame of 50x54 μm . A guard zone of 5 μm was used to avoid the artifacts of sectioning. The total number of DCX positive cells was estimated using the formula: $N_{(total)} = \Sigma Q^- \times 1/ssf \times \backslash A(x,y \text{ step}) / a(frame) \times t/h$; where ΣQ^- is the number of counted cells; ssf is the section sampling fraction; $A(x, y \text{ step})$ is the area associated with each x, y movement; $a(frame)$ is the area of the counting frame; t is the section thickness-weighted average, and h is the dissector height.

The dendritic morphology of immature granule neurons was quantified by categorizing a dendritic tree in a subset of DCX positive neurons from rats according to previously described methods (Lussier et al., 2013; Plümpe et al., 2006). A stereology system was used to randomly select at least a 100 cells per rat. The cells were placed into one of six categories depending on the presence and amount of the apical dendrites (see Figure 3-6, Panel B). Briefly, categories one and two cells had either no processes (category one) or very short processes (category two), and were considered as cells at the early proliferative stage of cell development. Category three cells had medium size processes that extended to the granular cell layer. Category four cells had a medium size process that extended to the molecular layer. Categories five and six cells were at more mature stages of development, with one major dendrite branching in the molecular layer (category five) or a well-defined

dendritic tree with several branches in the GCL (category six). The percentage of cells under each of these categories was determined for each rat.

3.2.5.2. REST

To visualize REST positive cells, free-floating tissue sections were first rinsed in 0.1 M TBS (pH 7.4). Following rinsing, the sections were subjected to heat-induced antigen retrieval in a sodium citrate buffer (pH 6.0) at 85 °C for 30 min. The sections were then blocked in a 0.5% (v/v) Triton X-100 TBS buffer containing 5% (v/v) normal goat serum (NHS) and 1% (w/v) bovine serum albumin (BSA; Sigma, USA) for 30 min at 37 °C, followed by incubation with a rabbit anti-REST polyclonal antibody (1:1500; generous gift from Dr. Jenny Hsieh, UT Southwestern Medical Center, USA) for 48 hours at 4 °C. Then, the sections were incubated in 1% H₂O₂ for 20 min to block endogenous peroxidase activity, followed by incubation with a biotinylated secondary antibody (goat anti-rabbit; 1:500; Sigma-Aldrich, USA) for 1.5 hours at 37 °C, and incubation in avidin-biotin complex (1:500, Vector Laboratories, USA) for 1.5 hours at 37 °C. Immunolabelling was visualized with 0.033% (w/v) DAB and 0.0078% (v/v) H₂O₂ diluted in TBS. After approximately 4 minutes, the sections were rinsed in TBS to stop the DAB reaction. The sections were then mounted onto glass slides, dehydrated, and coverslipped using Entellan resin (Millipore, USA) for light microscopic examination.

REST cell counts and optical density. The number of REST positive cells was quantified using a computerized image analysis program (Stereo Investigator, version 8.0, MicroBrightField Inc.) attached to a Nikon Eclipse E800 microscope. The following regions were quantified: hilus, CA3 strata oriens and radiatum, and CA1 strata oriens and radiatum, strata lacunosum. An experimenter who was blind to the treatment conditions manually counted cells using a profile counting method, which involved using a meander scan setting

to sample non-overlapping fields from each brain region. Five sections per brain were analyzed (from -2.40 mm from bregma to approximately -3.94 mm from bregma) in both right and left hemispheres. According to Schiffer et al. (2014), REST labelling is mainly cytoplasmic in neurons and nuclear in glial cells. Therefore, only large cells with dark, uniformly stained cytoplasm and well-defined cell bodies were counted as REST positive. The number of cells counted for each brain region, within the traced contours (in μm). The values were re-expressed as cells per mm^2 . Data are presented as the average number of REST+ cells per mm^2 within a defined contour within each region.

Optical density (OD) analysis was used to assess REST immunostaining in hippocampal subregions, where cells are normally tightly packed. This included the GCL, CA3 strata pyramidale and CA1 strata pyramidale. The dense aggregation of immunostained cells in these areas made accurate cell counting impossible, therefore OD analysis was conducted. Monochrome images of brain sections were collected at 200X magnification using a Nikon E800 microscope. Optical densities from 3 brain sections (both hemispheres) were determined using the Image J software program (v1.43u, National Institutes of Health). The OD was corrected according to the background staining measured in the corpus callosum of each individual section. The resulting average OD in each brain area was taken as a measure of REST immunoreactivity.

3.2.6. Statistical Analysis

Main effects and interactions were first analyzed using a two-way univariate ANOVA, with time and treatment as independent variables. Separate independent sample t-tests (one-tailed or two-tailed, as appropriate) were used to analyze treatment effect at specific experimental time points as per our *a priori* hypotheses. All data were analyzed

using Statistical Package for the Social Sciences (Chicago, IL, version 18.0). The criterion for statistical significance of between-group differences was set at $p < 0.05$.

Bivariate Pearson's correlations were used to examine the statistical relation between measures of depression-like behavior and neurobiological changes in CORT and vehicle groups of rats in treatment cycles one and three. The criterion for statistical significance of these correlational analyses was set at $p < 0.05$.

3.3. Results

3.3.1. Effects of Repeated and Cyclic CORT Treatment on Body Weight

Figure 3-2 illustrates the mean body weight for the rats in both groups at different time points throughout the experiment. The two-way ANOVA revealed a significant main effect of time [$F(8, 166) = 61.657, p < 0.001$] and treatment [$F(1, 166) = 37.92, p < 0.001$] on body weight. There was no significant interaction of treatment by time ($p = 0.521$).

In cycle 1, the CORT rats weighed significantly less than the vehicle rats at all time points: after 10 days of treatment, after 21 days of treatment, and after recovery [$t(18) = 3.299, p < 0.0001$; $t(19) = 1.864, p = 0.039$; $t(19) = 2.01, p = 0.029$, respectively]. Similarly, in cycle 2, the CORT rats also weighed significantly less than the vehicle rats after 10 and 21 days of treatment [$t(19) = 2.028, p = 0.029$; $t(18) = 2.84, p = 0.002$, respectively], but after recovery the difference between the CORT rats and vehicle rats narrowly missed statistical significance [$t(18) = 1.547, p = 0.069$]. Cycle 3 looked very much like cycle 1, with the CORT rats weighing significantly less than the vehicle rats at all time points [day 11: $t(18) = 1.935, p = 0.035$; day 22: $t(17) = 2.543, p = 0.011$; recovery: $t(20) = 1.749, p = 0.048$].

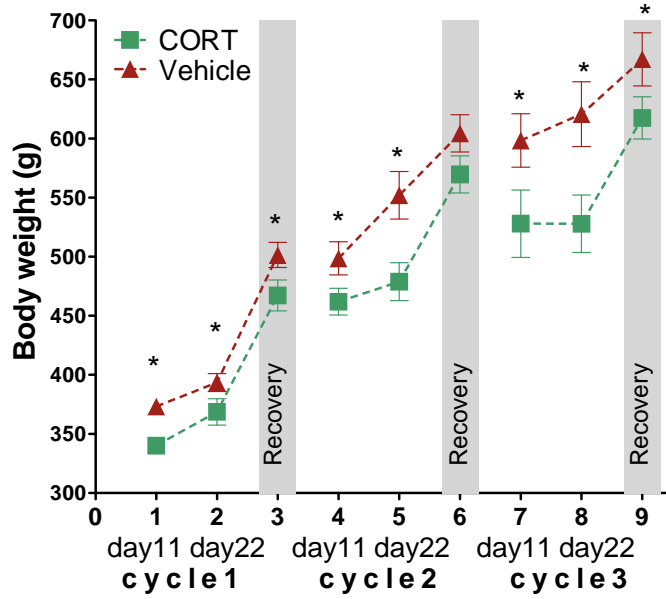


Figure 3-2. The effect of one, two, and three cycles of repeated corticosterone (CORT) injections on body weight. The CORT rats weighed less than the vehicle rats at all time points of the experiment. Asterisks (*) indicate significant differences between CORT and vehicle groups at specific time points ($p < 0.05$). Error bars denote the standard error of the mean (SEM).

3.3.2. Effects of Repeated and Cyclic CORT Treatment on Forced Swim Test Behaviors

Figure 3-3 shows the effects of cyclical CORT treatment on FST behaviors throughout the three cycles of treatment.

3.3.2.1. Immobility

The two-way ANOVA revealed a significant main effect of time [$F(8, 162) = 9.577, p < 0.001$] and treatment [$F(1, 162) = 20.304, p < 0.001$] on immobility. There was no significant interaction of treatment by time ($p = 0.174$).

In cycle 1, we found no significant difference between CORT and vehicle rats in the amount of time spent immobile after 10 days of treatment [$t(18) = 1.245, p = 0.115$]. However, after 21 days of treatment, the CORT rats spent significantly more time immobile than the vehicle rats [$t(19) = 2.249, p = 0.019$]. After the recovery period, the CORT and vehicle rats did not differ in the amount of time spent immobile [$t(19) = 0.136, p = 0.297$].

In cycle 2, the CORT rats spent significantly more time immobile than the vehicle rats after 10 days of treatment [$t(18) = 1.902, p = 0.037$], and continued to do so after 21 days of treatment [$t(18) = 2.946, p = 0.005$]. No statistical difference was found in immobility time between groups after the recovery period [$t(20) = 1.009, p = 0.163$].

Similarly, in cycle 3, the CORT rats spent significantly more time immobile compared to the vehicle rats after 10 days of treatment [$t(18) = 3.984, p = 0.0005$], and continued to do so after 21 days of treatment [$t(18) = 2.946, p = 0.005$]. No statistical difference was found in immobility between the groups after the recovery period [$t(20) = 1.009, p = 0.163$].

3.3.2.2. Latency to immobility

The two-way ANOVA revealed a significant main effect of time [$F(8, 162) = 5.368, p$

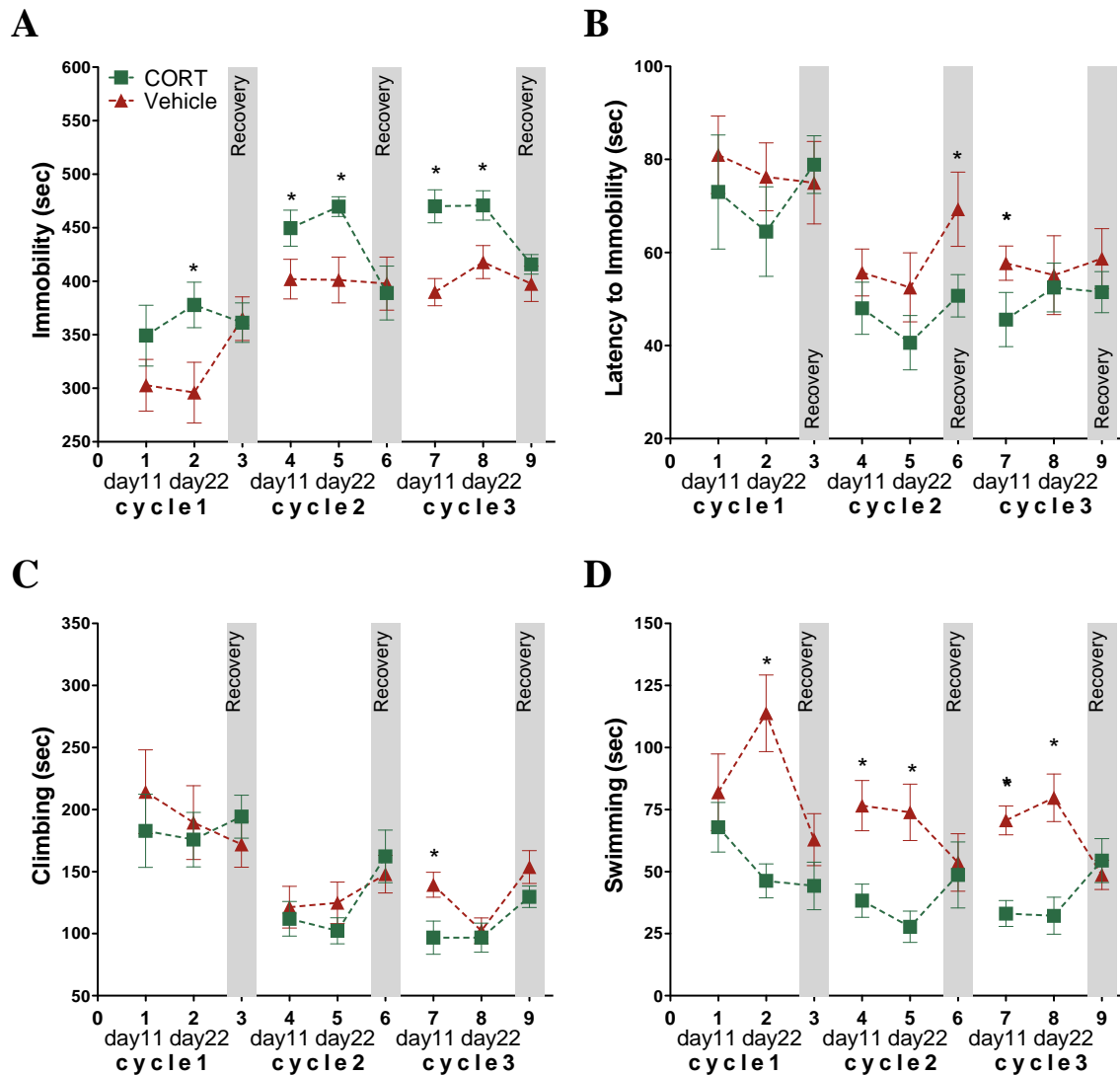


Figure 3-3. The effect of one, two, and three cycles of repeated corticosterone (CORT) exposure on behavior in the forced swim test (FST): immobility - panel (A), latency to immobility - panel (B), climbing - panel (C), and swimming - panel (D). CORT produced increases in immobility behavior and decreases in swimming behavior in all three treatment cycles. Rats treated with CORT spent significantly more time immobile and less time swimming and climbing at the end of treatment cycle one. Following CORT re-exposure in the second and third cycles, the CORT rats showed a sensitized increase in immobility and decrease in swimming after only ten days of treatment. Asterisks (*) indicate significant differences between CORT and vehicle groups at specific time points ($p < 0.05$). Error bars represent the standard error of the mean (SEM).

< 0.001] and treatment [$F(1, 162) = 6.335, p = 0.013$] on latency to immobility. There was no significant interaction of treatment by time ($p = 0.916$).

In general, there were few significant group differences in latency to immobility. All group differences were nonsignificant, except for two time points – recovery after cycle 2 [$t(14.27) = 2.027, p = 0.03$] and after 10 days of treatment in cycle 3 [$t(18) = 3.984, p = 0.042$]. In both of these cases, the CORT rats had a shorter latency to immobility than the vehicle rats. All other group differences were nonsignificant (all p values > 0.21).

3.3.2.3. Climbing

The two-way ANOVA revealed a significant main effect of time [$F(8, 162) = 6.964, p < 0.001$] on climbing. There was no significant main effect of treatment ($p = 0.166$) or interaction of treatment by time ($p = 0.773$).

No statistical differences between the groups were observed in climbing at any time point of the experiment (all p values > 0.18), with exception of cycle 3 (after 10 days of treatment) [$t(14.27) = 2.535, p = 0.01$].

3.3.2.4. Swimming

The two-way ANOVA revealed a significant main effect of time [$F(8, 162) = 2.477, p = 0.015$] and treatment [$F(1, 162) = 39.355, p < 0.001$] on swimming. Additionally, there was a significant interaction of treatment by time [$F(8, 162) = 2.216, p = 0.007$].

In cycle 1, we found no significant difference between the CORT and vehicle rats after 10 days of treatment ($p > 0.05$). However, after 21 days of treatment, the CORT rats showed significantly less swimming than did the vehicle rats [$t(13.75) = 4.00, p = 0.001$]. After the recovery period, CORT and vehicle rats did not differ in the amount of time spent swimming ($p = 0.209$).

In cycle 2, the CORT rats swam significantly less than vehicle rats after ten days of treatment [$t(15.605) = 3.157, p = 0.003$], and continued to do so after 21 days of treatment [$t(14.107) = 3.539, p = 0.001$]. No statistical difference between the groups was found after the recovery period ($p = 0.78$).

Similarly, in cycle 3, the CORT rats swam significantly less than the vehicle rats after 10 days of treatment [$t(18) = 4.796, p = 0.001$], and continued to do so after 21 days of treatment [$t(14) = 3.917, p = 0.001$]. No statistical group difference was found in swimming after the recovery period ($p = 0.608$).

3.3.3. Effects of Repeated and Cyclic CORT Treatment FST Behaviors Controlled for Body Weight

To account for the influence of body weight increases on FST behaviors, ratio scores were calculated and statistically analyzed. $T/BW = \text{ratio score}$, where T is the time the subject spent engaging in FST behaviors (immobility, climbing, etc.) in seconds, and BW is rat's body weight in grams at the time of testing. Figure 3-4 shows the mean ratio scores in each treatment group for the FSTs conducted during cycle 1 through 3.

3.3.3.1. Immobility to Body Weight (IBW) Ratio

The two-way ANOVA revealed a significant main effect of time [$F(8, 162) = 9.141, p < 0.001$] and treatment [$F(1, 162) = 51.652, p < 0.001$] on immobility/body weight (IBW) ratio. Additionally, there was a significant interaction of treatment by time [$F(8, 162) = 1.953, p = 0.05$].

CORT treatment had significant effects on the IBW ratio during all three cycles. In cycle 1, 20 mg/kg CORT rats showed smaller ratio scores than did the vehicle rats in both FST-1 [$t(18) = 2.04, p = 0.05$] and FST-2 [$t(19) = 2.4, p = 0.027$], after 10 and 21 days of

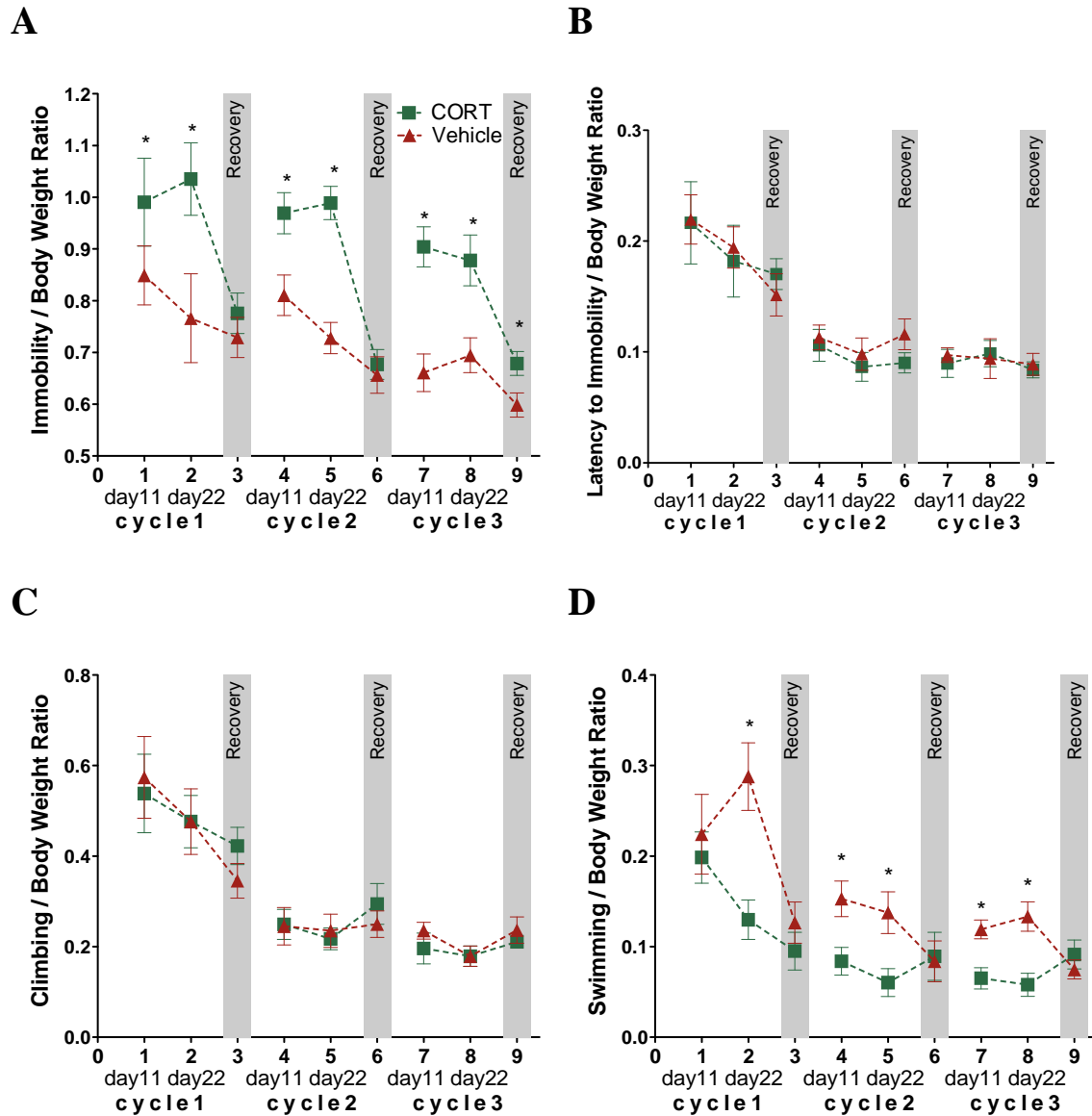


Figure 3-4. The effect of repeated and cyclic corticosterone (CORT) on the forced swim test (FST) behavior plotted by body weight. Panel (A) shows the immobility time to body weight ratio, panel (B) shows the latency to immobility to body weight (IBW) ratio, panel (C) shows the climbing time to body weight ratio, and panel (D) shows the time spent swimming to body weight (SBW) ratio. Rats treated with CORT exhibited larger IBW ratio and lower SBW scores at the end of treatment cycle one. Following CORT re-exposure in the second and third cycles, the CORT rats showed a sensitized increase in the IBW scores and decreases in SBW scores after only ten days of treatment. Asterisks indicate significant differences between groups at specific time points ($p < 0.05$). Error bars denote the standard error of the mean (SEM).

treatment, respectively. No significant group differences were found after recovery on FST-3 ($p = 0.409$).

Similarly, in cycle 2, 20 mg/kg CORT rats had lower IBW ratio scores than the vehicle rats after 10 days of treatment on FST-4 [$t(18) = 2.85, p = 0.011$], and after 21 days of treatment on FST-5 [$t(18) = 5.94, p < 0.001$]. No significant group differences were found after recovery on FST-6 ($p = 0.656$).

In cycle 3, 20 mg/kg CORT rats showed lower IBW ratio scores than did the vehicle rats after 10 days and 21 days of treatment on FST-7 [$t(18) = 4.6, p < 0.001$] and on FST-8 [$t(14) = 3.085, p = 0.008$]. Additionally, a significant difference between groups on IBW ratios was found in FST-9, after the recovery [$t(20) = 2.43, p = 0.024$].

3.3.3.2. Latency to Immobility to Body Weight (LIBW) Ratio

The two-way ANOVA revealed a significant main effect of time [$F(8, 162) = 15.395, p < 0.001$] on latency to immobility/body weight (LIBW) ratio. There was no main effect of treatment ($p = 0.518$), or interaction of treatment by time ($p = 0.982$).

No statistical differences between the groups were observed in LIBW ratios at any time point of the experiment (all p values > 0.138).

3.3.3.3. Climbing to Body Weight (CBW) Ratio

The two-way ANOVA revealed a significant main effect of time [$F(8, 162) = 15.701, p < 0.001$] on climbing/body weight (CBW) ratio. There was no main effect of treatment ($p = 0.984$), or interaction of treatment by time ($p = 0.949$).

No statistical differences between the groups were observed in CBW ratios at any time point of the experiment (all p values > 0.19).

3.3.3.4. Swimming to Body Weight (SBW) Ratio

The two-way ANOVA revealed a significant main effect of time [$F(8, 162) = 9.686, p < 0.001$] and treatment [$F(1, 162) = 23.742, p < 0.001$] on swimming/body weight (SBW) ratio. Additionally, there was a significant interaction of treatment by time [$F(8, 162) = 2.127, p = 0.012$].

In cycle 1, we found no significant difference between the CORT and vehicle rats after ten days of treatment ($p = 0.629$). However, after 21 days of treatment, the CORT rats had significantly lower SBW ratios than did the vehicle rats [$t(15.9) = 3.67, p = 0.002$]. After the recovery period, CORT and vehicle rats did not differ in SBW ratios ($p = 0.321$).

In cycle 2, the CORT rats showed significantly lower SBW ratios than did vehicle rats after 10 days of treatment [$t(18) = 2.78, p = 0.012$], and continued to do so after 21 days of treatment [$t(18) = 2.79, p = 0.012$]. No statistical difference between the groups was found after the recovery period ($p = 0.92$).

Similarly, in cycle 3, the CORT rats had significantly smaller SBW ratios than the vehicle rats after 10 days of treatment [$t(18) = 3.48, p = 0.003$], as well as after 21 days of treatment [$t(14) = 3.63, p = 0.003$]. No statistical group difference was found in SBW ratios after the recovery period ($p = 0.395$).

3.3.4. Assessment of Recovery of Immobility Behavior Across Three Cycles of CORT Treatment

To evaluate the quality of recovery across three cycles of CORT treatment, the percent change in immobility time and IBW ratio in the CORT rats were calculated. In the present experiment, obtaining the individual values of the percent recovery is not possible due to the non-repeated measures design (no pairwise comparisons). Therefore, for calculations, we obtained the difference in the group mean scores in the FST on day 22 and after recovery, and then divided that amount by the day 22 FST mean score, and multiplied

by 100. The analogous calculations were carried out for cycles two and three. The results are listed in Table 3-1.

3.3.5. Effects of Repeated and Cyclic CORT Treatment on Open Field Test Behavior

Figure 3-5A shows the effect of CORT on behavior in the OFT. The two-way ANOVA revealed a significant main effect of time [$F(8, 166) = 5.149, p < 0.001$] on total OFT activity (the number of total lines crossed and rearing counts). There was no main effect of treatment ($p = 0.204$) or interaction of treatment by time ($p = 0.861$).

The separate t-test analyses confirmed no significant differences between the groups in total OFT activity at any time point of the experiment (all p values > 0.094).

3.3.6. Effects of Repeated and Cyclic CORT Treatment on Sucrose Preference Test Behavior

Figure 3-5B illustrates the effect of CORT on behavior in the SPT throughout the three cycles of treatment and recovery. The two-way ANOVA revealed a significant main effect of time [$F(8, 154) = 1.993, p = 0.05$] and treatment [$F(1, 154) = 10.117, p = 0.002$] on sucrose preference. There was no significant interaction of treatment by time ($p = 0.231$).

In cycle 1, we found no significant difference between the CORT and vehicle rats in sucrose preference at 10 or 21 days of treatment. Similarly, no difference in sucrose preference was observed after the recovery period [all p values > 0.25].

In cycle 2, no statistically significant group difference was observed in sucrose preference after 10 days of treatment [$t(17) = 1.094, p = 0.145$]. After 21 days however, the CORT rats had a significantly reduced preference for sucrose [$t(17) = 2.525, p = 0.015$]. After recovery, sucrose preference was similar in both groups [$t(16) = 0.744, p = 0.234$]. Similarly, in cycle 3, no statistically significant group difference was observed in sucrose

Table 3-1. Changes in recovery over three cycles of corticosterone (CORT) treatment. The listed values are the percent change in the group mean immobility time and IBW ratio in the 20 mg/kg CORT group. For details see the text.

	Immobility Time, % change	Immobility to Body Weight Ratio, % change
Recovery, Cycle 1	4.4%	25.1%
Recovery, Cycle 2	17.2%	31.5%
Recovery, Cycle 3	11.7%	22.7%

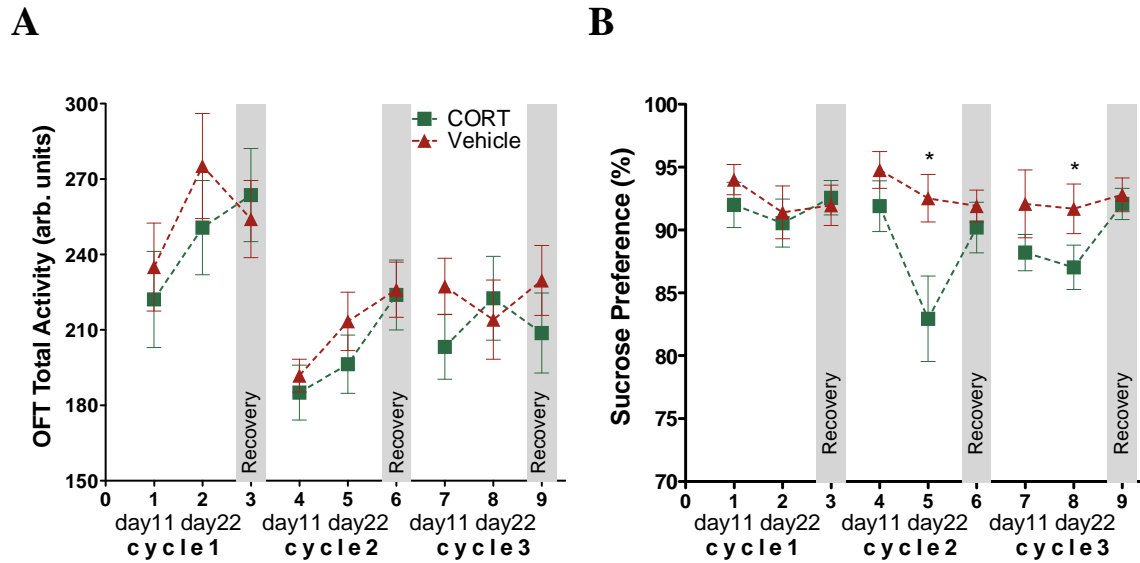


Figure 3-5. The effect of one, two, and three cycles of repeated corticosterone (CORT) exposure on behavior in the open field (A) and in the sucrose preference test (B). CORT produced significant decreases in sucrose preference after 21 days of treatment in cycles two and three, but not in cycle one. There were no group differences in general locomotor behavior. Asterisks (*) denote a significant difference between the CORT and vehicle groups at specific time points ($p < 0.05$). Error bars represent the standard error of the mean (SEM). Arb. units, arbitrary units; OFT, open field test.

preference after 10 days of treatment [$t(15) = 1.253, p = 0.118$], but after 21 days, the CORT rats had a reduced preference for sucrose [$t(16) = 1.883, p = 0.039$]. Again, sucrose preference was similar in both groups after recovery [$t(18) = 0.409, p = 0.334$].

3.3.7. Effects of Repeated and Cyclic CORT Treatment on the total DCX+ cell number in SGZ and GCL

Figure 3-6 shows the effect of CORT on the number of immature granule neurons in the SGZ and GCL. The two-way ANOVA revealed a significant main effect of time [$F(8, 73) = 33.87, p < 0.001$] and treatment [$F(1, 73) = 32.092, p < 0.001$] on the number of DCX+ cells in SGZ and GCL. There was no significant interaction of treatment by time ($p = 0.26$).

In cycle 1, the CORT had fewer DCX positive cells in the SGZ and GCL on days 11 and 22 compared to the vehicle rats, but these differences narrowly missed statistical significance [all p values > 0.07]. After the recovery period, the CORT rats did have significantly fewer DCX positive cells than did the vehicle rats [$t(9) = 3.471, p = 0.004$].

In cycle 2, no significant differences were observed between CORT and vehicle rats in the number of DCX positive cells after ten days of treatment [$t(8) = 1.459, p = 0.092$]. However, after 21 days of treatment, the CORT rats had significantly fewer DCX positive cells compared to the vehicle rats [$t(8) = 2.739, p = 0.013$]. This trend continued after recovery, but the group differences narrowly missed statistical significance [$t(8) = 1.659, p = 0.068$].

In cycle 3, no differences were observed between the CORT and vehicle rats in the number of DCX positive cells after 10 days of treatment [$t(8) = 1.457, p = 0.092$]. After 21 days of treatment, the CORT rats had significantly fewer DCX positive cells compared to the vehicle rats [$t(8) = 2.76, p = 0.013$]. After recovery, no statistical difference was found between groups [$t(8) = 0.515, p = 0.621$].

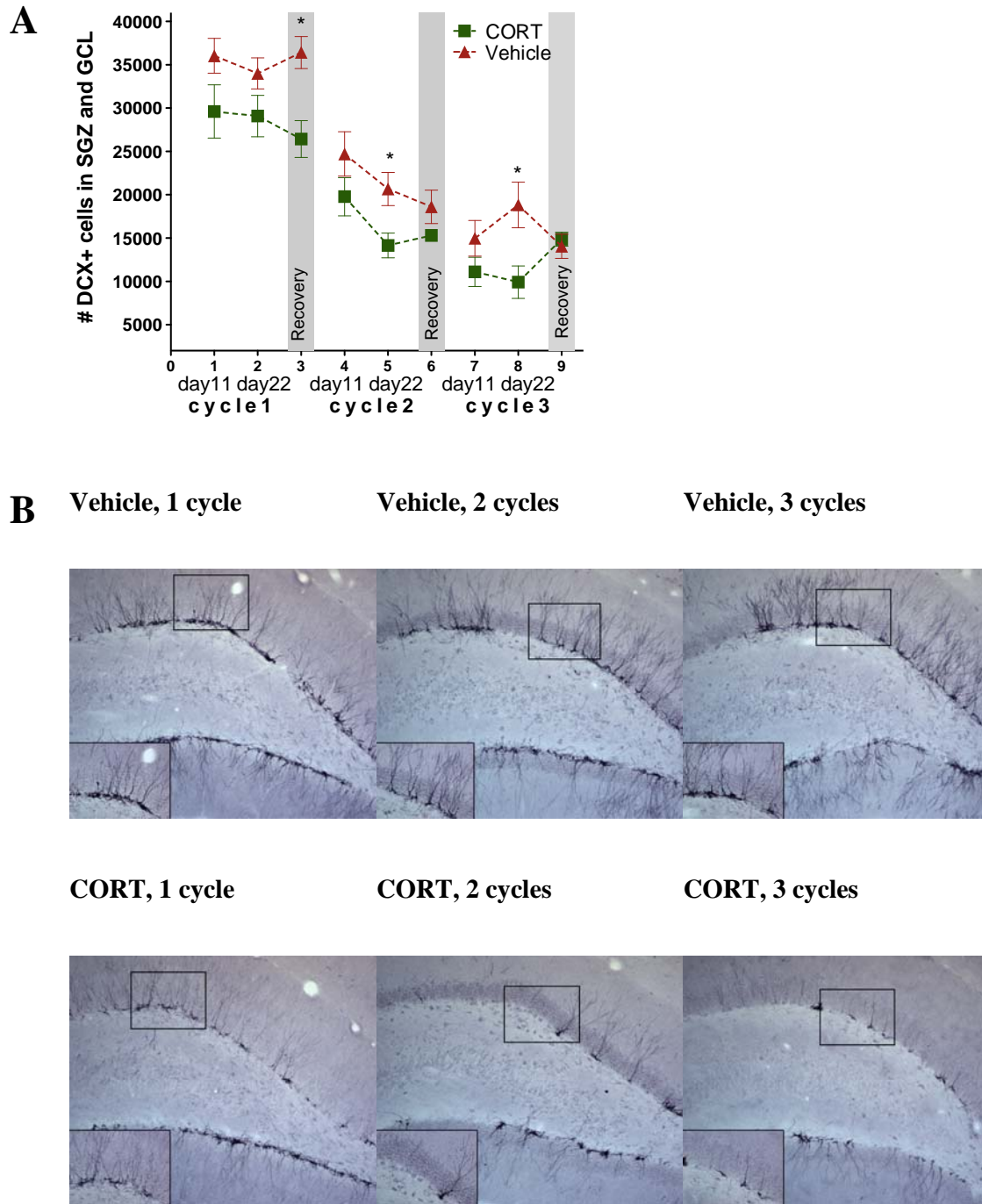


Figure 3-6. The effect of one, two, and three cycles of repeated corticosterone (CORT) treatment on the number of doublecortin (DCX)-positive cells in the subgranular zone (SGZ) and granule cell layer (GCL): Panel (A) shows the number of DCX-positive cells in the CORT and vehicle groups at different points of CORT exposure and recovery. Panel (B) shows representative micrographs of DCX immunoreactivity at the end of each cycle of CORT treatment. CORT treatment produced accumulative decreases in the number of DCX-positive cells in SGZ and GCL. Error bars represent the standard error of the mean (SEM). Asterisks (*) denote statistical significance between groups at specific time points ($p < 0.05$).

3.3.7.1. Effects of Repeated and Cyclic CORT Treatment on Maturation of DCX+ neurons in the Subgranular Zone and Granule Cell Layer

To assess the effect of CORT on the maturation rate of newborn granule cells, we examined dendritic complexity in a subset of DCX positive cells in rats treated for one cycle (CORT and vehicle) and rats treated for three cycles (CORT and vehicle). Each DCX positive cell was placed into a category based on the presence and extent of dendritic branching (Figure 3-7B). The results of this analysis are shown in Figure 3-7A. The two-way ANOVA analyses revealed no main effect of time on the percentage of category one, two, three, five, and six DCX+ cells (all p values > 0.09), however there was a significant main effect of time on the percentage of category four DCX+ cells [$F(1,15) = 4.647, p = 0.048$]. No main effect of treatment was found on the percentage of category one DCX+ cells ($p = 0.411$), however the significant main effects of treatment were observed on the percentage of category two [$F(1,15) = 8.187, p = 0.012$], category three [$F(1,15) = 9.551, p = 0.007$], category four [$F(1,15) = 8.758, p = 0.010$], category five [$F(1,15) = 38.922, p < 0.001$], and category six DCX+ cells [$F(1,15) = 16.174, p = 0.001$]. No significant interactions of time by treatment were found (all p values > 0.233).

Separate t-test analyses at specific time points revealed, in cycle 1, CORT rats had a significantly larger percentage of category three and four cells compared to the vehicle rats [$t(8) = 1.967, p = 0.043$; $t(8) = 2.878, p = 0.019$, respectively]. However, the CORT rats had significantly fewer category five and six DCX positive cells [$t(8) = 3.531, p = 0.007$; $t(8) = 3.268, p = 0.012$]. In cycle 3, the CORT rats had a significantly higher percentage of category two DCX positive cells [$t(7) = 4.823, p = 0.001$], and significantly lower percentage of category five cells [$t(7) = 4.596, p = 0.001$] and category six cells [$t(4.85) = 3.326, p = 0.022$] compared to the vehicle rats.

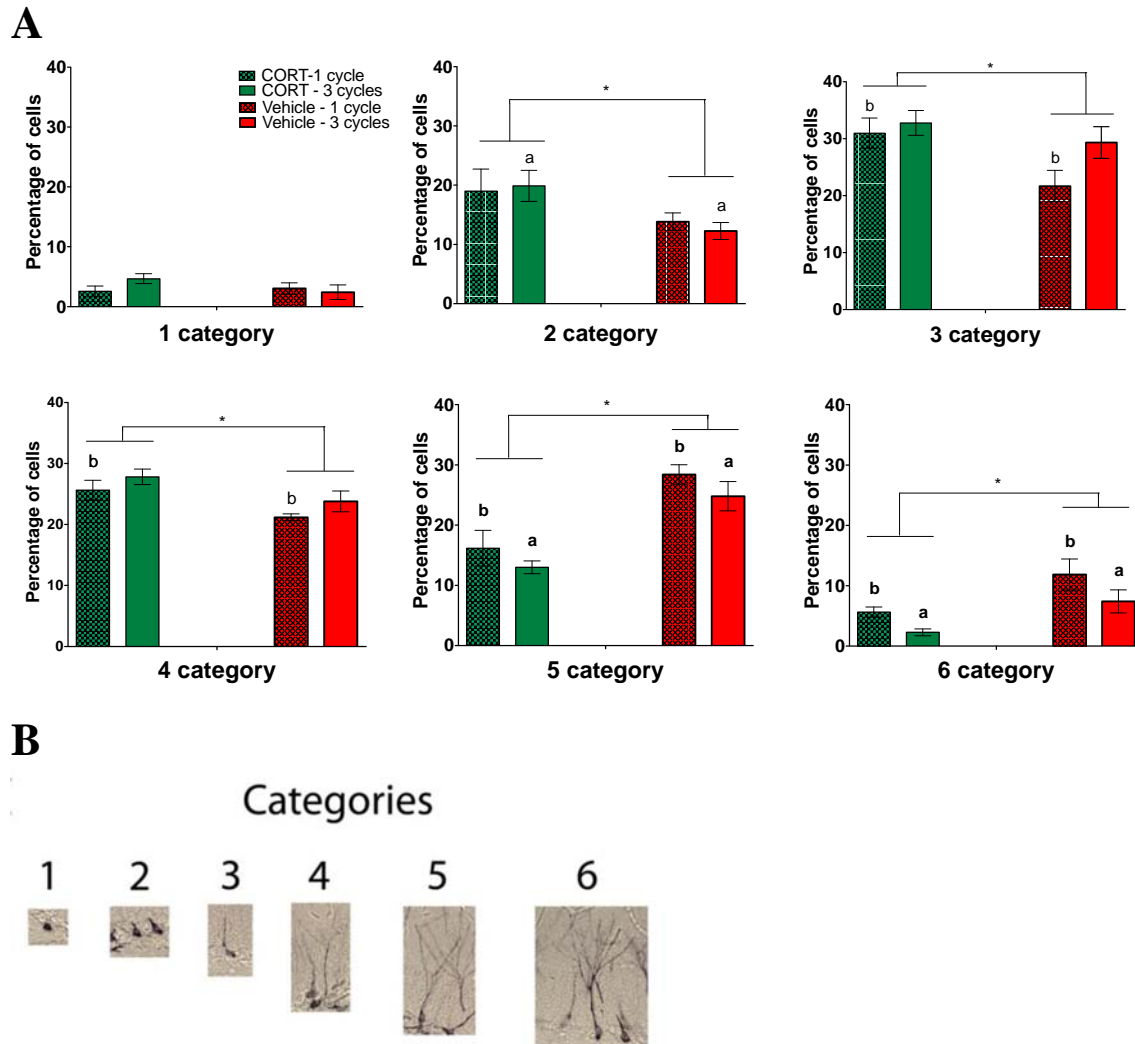


Figure 3-7. The effect of one and three cycles of repeated corticosterone (CORT) treatment on the maturation of doublecortin (DCX)-positive cells in the subgranular zone (SGZ) and granule cell layer (GCL): Panel (A) shows the percentage of cells placed into each category in the CORT and vehicle groups, treated for one or three cycles. Panel (B) provides an example the six categories of neuron maturation stages used in this analysis (image adapted from Lussier et al., 2013). CORT treatment affected the percentage of cells in categories two, five, and six, with a total increase in the percentage of category two DCX-positive cells (immature stage of development) and a total decrease in the percentage of category five and six cells (more mature stages of development). Error bars denote the standard error of the mean (SEM). Asterisks (*) indicate overall significant differences between CORT and vehicle groups; letters (a) and (b) indicate significant differences between two vehicle groups (treated with one and three cycles) and CORT groups (treated with one and three cycles), respectively ($p < 0.05$).

3.3.8. Effects of Repeated and Cyclic CORT Treatment on REST Levels in the Hippocampus

3.3.8.1 REST Expression Levels in CA Pyramidal and Granule Cell Layers

Figure 3-8 illustrates the effect of CORT on REST immunoreactivity in several hippocampal regions in cycles one and three. The two-way ANOVA analyses revealed a significant main effect of time on REST optical density in CA1 and CA3 pyramidal cell layers [$F(1,14) = 5.278, p = 0.038$ and $F(1,14) = 7.779, p = 0.014$, respectively], but not in the GCL ($p = 0.084$). No significant main effects of treatment were found on REST optical density in CA1, CA3 pyramidal cell layers, or GCL (all p values > 0.361). There was a significant interaction of time by treatment on REST optical density in GCL [$F(1,14) = 5.377, p = 0.036$].

The treatment effects at specific time points of the experiment were analyzed using separate t-tests. In cycle 1, the CORT rats had a small increase in REST expression in the CA1 and CA3 pyramidal layers and the GCL compared to the vehicle rats, but the difference did not reach statistical significance (all p values > 0.414). In cycle 3, the CORT rats had decreased REST expression in the CA3 pyramidal layer [$t(7) = 2.562, p = 0.037$] and GCL [$t(7) = 3.860, p = 0.006$] compared to the vehicle rats. There were no group differences in the CA1 pyramidal layer ($p = 0.365$).

Within the control rats, vehicle rats treated for three cycles had a significant increase in REST expression in the GCL, CA1 and CA3 pyramidal layers [$t(8) = 3.359, p = 0.01$; $t(8) = 3.906, p = 0.005$; $t(5.14) = 3.452, p = 0.017$, respectively]. No significant differences in these hippocampal subregions were found between CORT rats treated for one and three cycles (all p values > 0.541).

3.3.8.2. REST+ Cell Counts in Hippocampal Subregions

Figure 3-9 illustrates the effect of CORT on the number of REST positive cells in

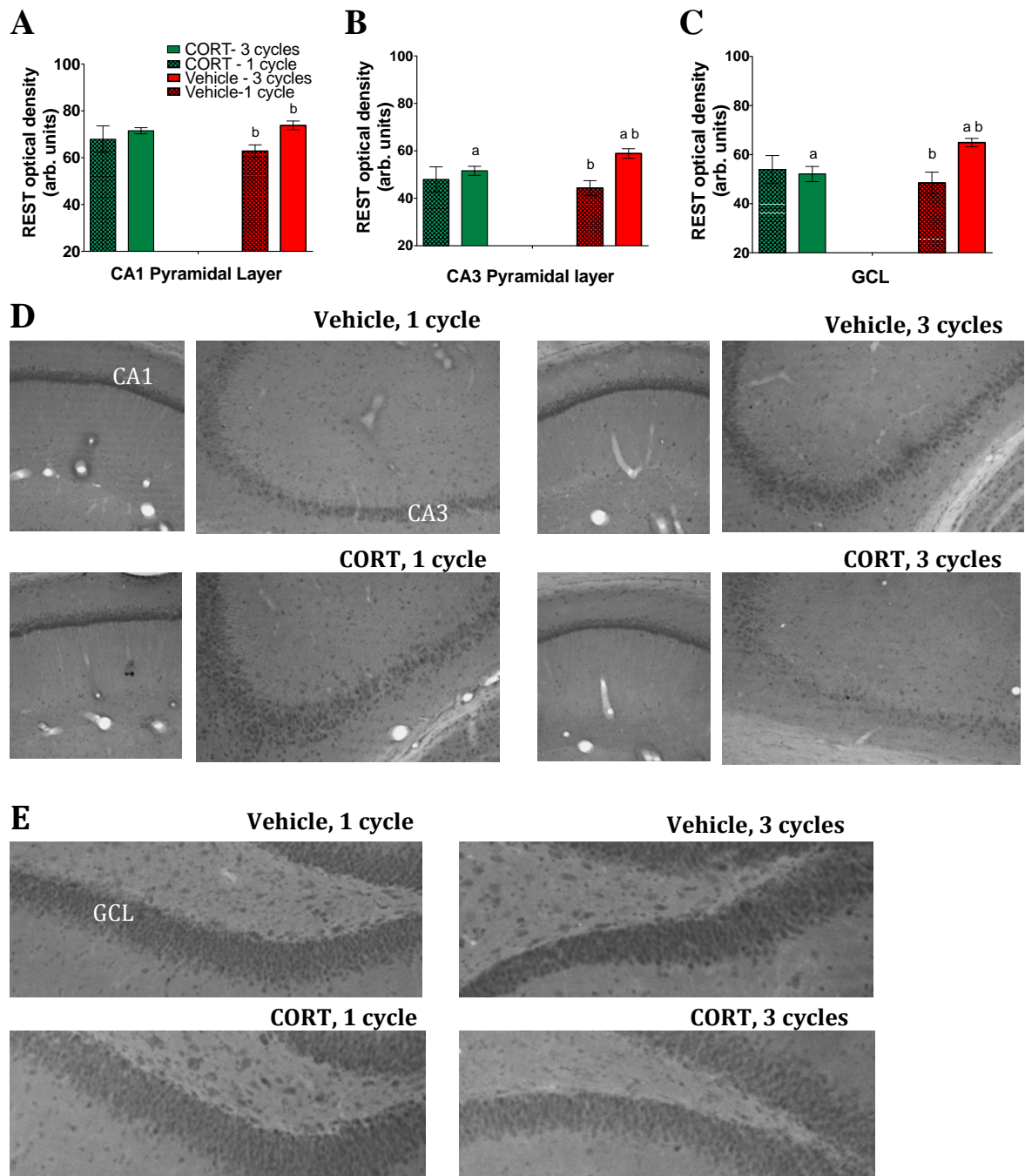


Figure 3-8. The effect of one and three cycles of repeated corticosterone (CORT) treatment on RE-1 Silencing Transcription factor (REST) protein density in several hippocampal regions: Panel (A) shows quantified REST levels in the hippocampal CA1 pyramidal cell layer, Panel (B) – in the CA3 pyramidal cell layer, and Panel (C) in the granule cell layer (GCL). Representative photomicrographs of REST protein density in the CA1 and CA3 (Panel (D)) pyramidal cell layers, and GCL (Panel (E)) in CORT and vehicle rats for one or three cycles. REST optical density was decreased in CORT rats compared to vehicle rats in the CA3 pyramidal cell layer and GCL in cycle three. Error bars denote the standard error of the mean (SEM). Letter (a) denotes significant differences between CORT and vehicle groups treated for three cycles; letter (b) denotes differences between two vehicle groups (treated for one and three cycles) ($p < 0.05$).

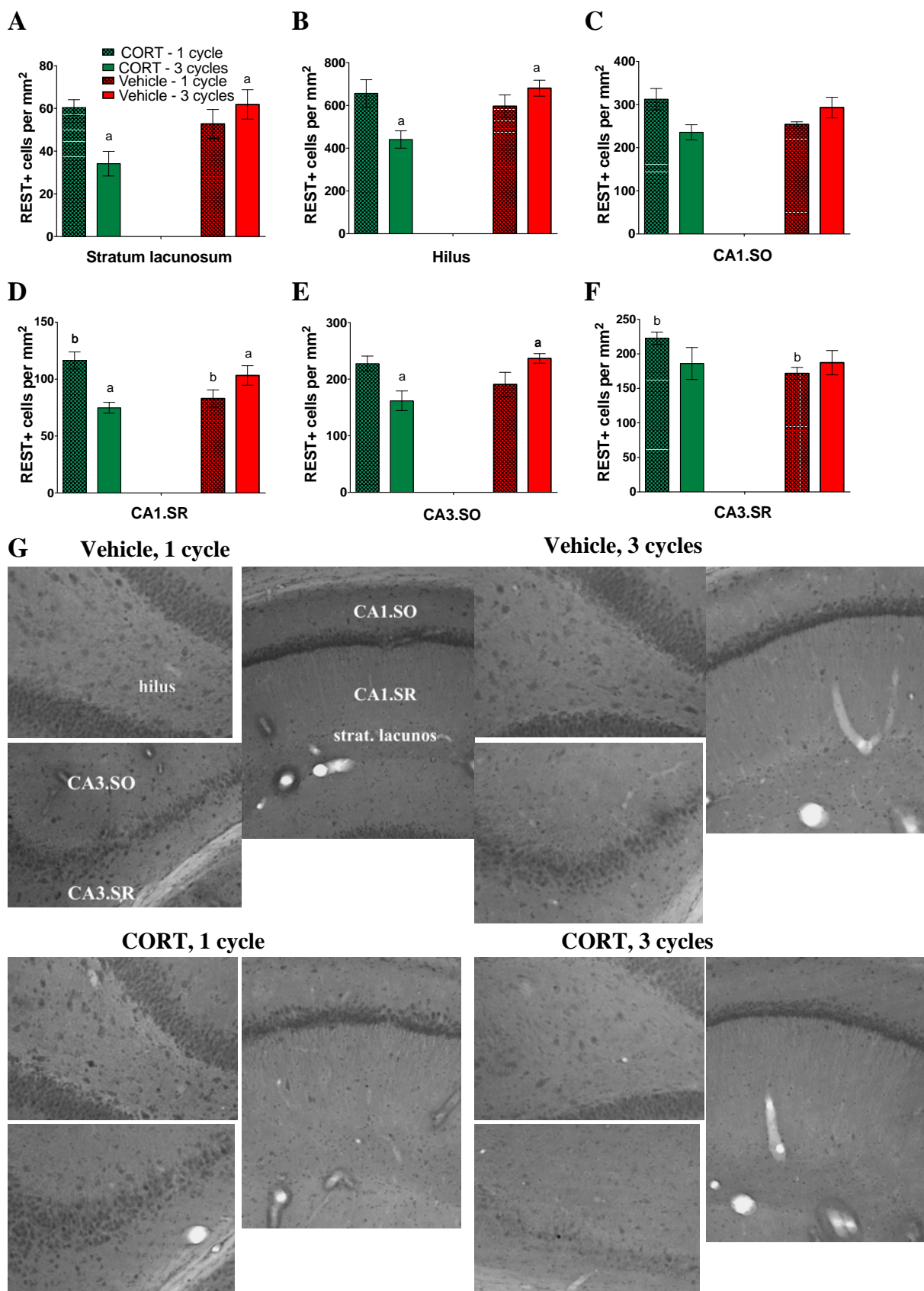


Figure 3-9. The effect of one and three cycles of repeated corticosterone (CORT) treatment on the number of RE-1 Silencing Transcription factor (REST)-positive cells in several hippocampal regions: Panel (A) the stratum lacunosum, Panel (B) the hilus, Panel (C) the CA1 stratum oriens (SO), Panel (D) the CA1 stratum radiatum (SR), Panel (E) the CA3 stratum oriens (SO), and Panel (F) the CA3 stratum radiatum (SR). Panel (G) shows representative photomicrographs of REST immunohistochemistry in these hippocampal regions from CORT and vehicle rats treated for one and three cycles. Bi-directional changes in REST expression were observed in several hippocampal subregions, with overall increases in REST+ cell counts after one cycle of CORT treatment and overall decreases after three cycles of CORT treatment. Error bars denote the standard error of the mean (SEM). Letters (a), and (b) represent significant differences between CORT and vehicle groups treated for three cycles and one cycle, respectively ($p < 0.05$).

several hippocampal subregions in cycles one and three. The two-way ANOVA analyses revealed no significant main effects of time or treatment on the total number of REST+ cells in hilus, stratum lacunosum, CA1 strata radiatum and oriens, and CA3 strata radiatum and oriens (all p values > 0.116). However, significant interactions of time by treatment were observed on the number of REST+ cells in hilus [$F(1,15) = 8.479, p = 0.011$], stratum lacunosum [$F(1,15) = 8.715, p = 0.01$], CA1 stratum oriens [$F(1,15) = 8.421, p = 0.011$], CA1 stratum radiatum [$F(1,15) = 16.625, p = 0.001$], and CA3 stratum oriens [$F(1,15) = 12.531, p = 0.003$].

The treatment effects at specific time points of the experiment were analyzed using separate t-tests. In cycle 1, the CORT rats had a significant increase in the number of REST positive cells in the CA1 stratum radiatum [$t(8) = 3.126, p = 0.014$] and CA3 stratum radiatum [$t(8) = 4.101, p = 0.003$] compared to the vehicle rats. A similar increase was observed in other quantified regions of hippocampus, but the group differences did not reach statistical significance (all p values > 0.07).

In contrast to the results for cycle 1, in cycle 3 the CORT rats had fewer REST positive cells than the vehicle rats in the hilus [$t(7) = 4.317, p = 0.003$], the stratum lacunosum [$t(7) = 2.979, p = 0.021$], the CA1 stratum radiatum [$t(7) = 2.662, p = 0.032$], and the CA3 stratum oriens [$t(7) = 4.175, p = 0.004$].

3.3.9. Analyses of Correlations

Figure 3-10 depicts a number of correlations between measures of depression-like behavior and neurobiological changes in the CORT and vehicle rats in cycles one and three.

3.3.9.1. Correlations between Behavioral Measures and DCX+ Cell Counts and Maturation Rate

Pearson's correlation analyses revealed that the time spent immobile was strongly and

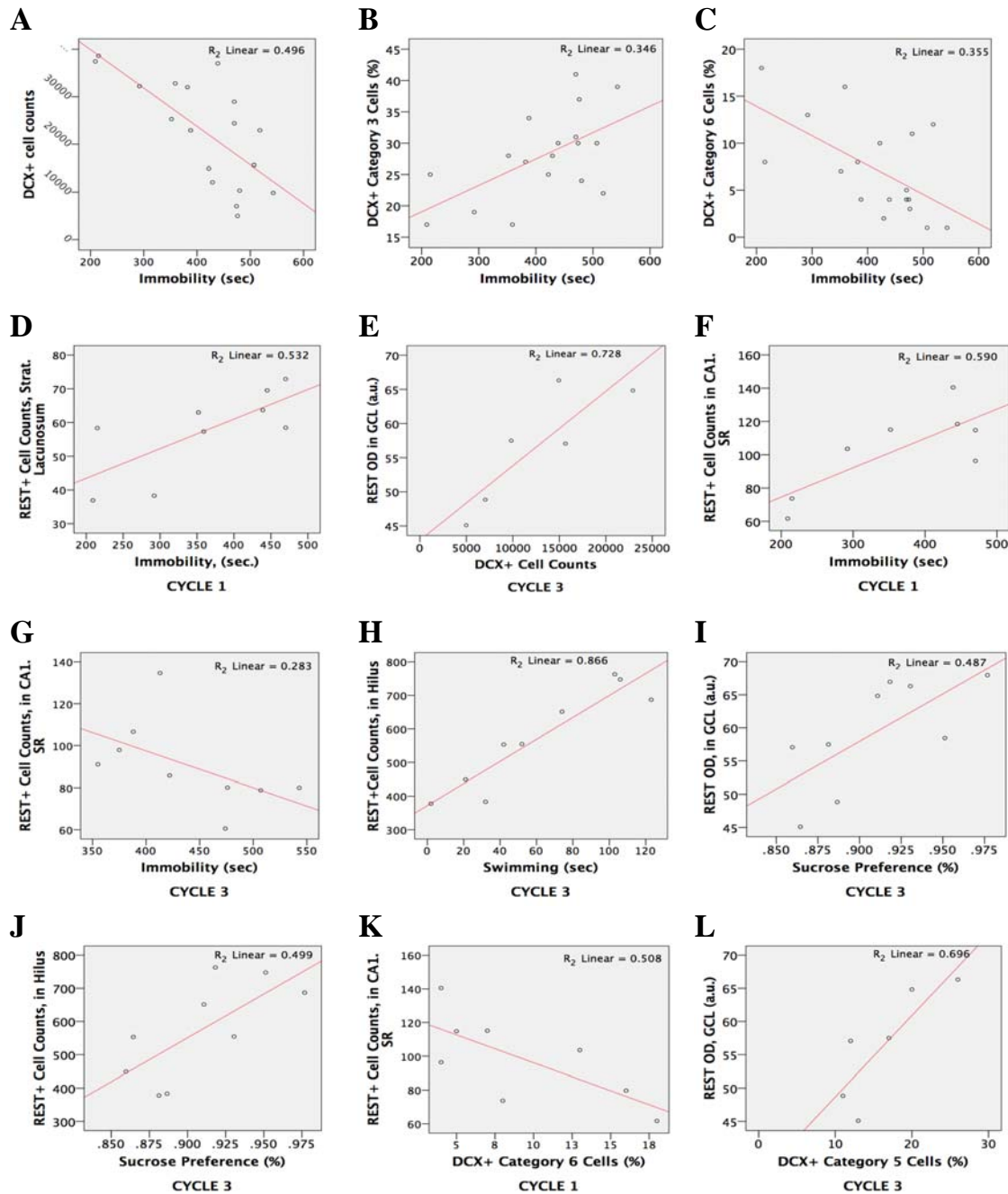


Figure 3-10. Correlations between measures of depression-like behavior and neurobiological changes in the corticosterone (CORT) and vehicle rats in cycles one and three. Correlation of immobility behavior with the total number of doublecortin (DCX)-positive cells in the granule cell layer (GCL) and subgranular zone (SGZ), ($r = -0.705$, $p = 0.001$) (A), with the percentage of DCX-positive category three cells ($r = 0.577$, $p = 0.012$) (B), and with the percentage of DCX-positive category six cells ($r = -0.596$, $p = 0.009$) (C). Correlation of immobility behavior with the number of RE-1 Silencing Transcription factor (REST)-positive cells in the stratum lacunosum, in cycle one ($r = 0.730$, $p < 0.05$) (D). Correlation of the total number of DCX-positive cells in GCL and SGZ with REST optical density in the GCL in cycle three ($r = 0.853$, $p < 0.05$) (E). Correlation of immobility behavior with the number of REST-positive cells in the CA1 stratum radiatum in cycle one ($r = 0.733$, $p < 0.05$) (F), with

the number of REST-positive cells in the CA1 stratum radiatum in cycle three ($r = 0.532, p < 0.05$) (G). Correlation of the number of REST-positive cells in the hilus with swimming in cycle three (H). Correlation of sucrose preference with REST optical density in the GCL in cycle one (I) and with REST optical density in the hilus in cycle three ($r = 0.931, p < 0.001$) (J). Correlation of the number of REST-positive cells in the CA1 stratum radiatum with the percentage of DCX-positive category six cells in cycle one ($r = -0.740, p < 0.05$) (K). Correlation of REST optical density in the GCL with the percentage of DCX-positive category five cells in cycle three ($r = 0.827, p < 0.05$) (L).

inversely associated with the number of DCX+ cells in GCL and SGZ (Figure 3-10A; $r = -0.705$, $p = 0.001$, $N = 18$). Additionally, immobility was positively correlated with the percentage of category three DCX+ cells (Figure 3-10B; $r = 0.577$, $p = 0.012$, $N = 18$), and negatively correlated with the percentage of category six DCX+ cells (Figure 3-10C; $r = -0.596$, $p = 0.009$, $N = 18$). Swimming behavior and latency to immobility were positively correlated with the number of DCX+ cells in GCL and SGZ ($r = 0.748$ and $r = 0.526$, respectively; $p = 0.001$, $N = 18$).

3.3.9.2. Correlations between REST (Cell Counts and Optical Density) and DCX+ Cell Counts and Maturation Rate

Due to bi-directional changes in REST, correlational analyses involving REST were conducted separately for treatment cycle 1 and cycle 3. In cycle 3, REST protein optical density in the GCL (Figure 3-10E), CA3 and CA1 pyramidal cell layers was positively correlated with the number of DCX+ cells ($r = 0.853$, $r = 0.835$, and $r = 0.962$, $p < 0.05$, $N = 6$, respectively). Additionally, the number of DCX+ cells in the stratum lacunosum was correlated with the number of DCX+ cells ($r = 0.969$, $p = 0.001$, $N = 7$).

With regard to neuronal maturation, in cycle 1 there was a significant positive correlation between the number of REST+ cells in the CA1 stratum oriens and the percentage of category three cells in the GCL and SGZ ($r = 0.763$, $p < 0.05$, $N = 8$). In contrast, the percentage of category six DCX+ cells in the GCL and SGZ was inversely correlated with the number of REST+ cells in CA1 stratum radiatum (Figure 3-10K) and CA3 stratum radiatum ($r = -0.740$, and $r = -0.760$, $p < 0.05$, $N = 8$, respectively). In cycle 3, the percentage of category five DCX+ cells was positively correlated with REST optical density in the GCL (Figure 3-10L; $r = 0.827$, $p < 0.05$, $N = 7$).

3.3.9.3. Correlations between REST (Cell Counts and Optical Density) and Behavioral Measures

Sucrose preference was positively correlated with REST optical density in the GCL (Figure 3-10I) and with the number of REST+ cells in the hilus (Figure 3-10J) in cycle 3 only ($r = 0.698$, and $r = 0.706$, $p < 0.05$, $N = 9$, respectively). Finally, the time spent immobile was positively correlated with the number of REST+ cells in cycle 1 (Table 3-1), and inversely correlated with the number of REST+ cells in cycle 3 (Table 3-2). Conversely, time spent swimming was positively correlated with the number of REST+ cells in hilus, in cycle 3 (Figure 3-10H; $r = 0.931$, $p < 0.001$, $N = 9$).

3.4. Discussion

The results of this experiment can be summarized as follows. We found that 3 cycles of repeated CORT administration produced time-dependent increases in depression-like behavior and decreases in neurogenesis that are consistent with the results of previous experiments (Lussier et al., 2013; review in Sterner & Kalynchuk, 2010). In addition, CORT had significant effects on dendritic complexity in newborn dentate granule neurons, indicating that cell maturation is dysregulated in rats exposed to repeated treatment with glucocorticoids. Finally, CORT altered REST protein immunoreactivity in various regions of the hippocampus, providing evidence of altered transcriptional regulation. These findings are discussed in more detail in the following paragraphs.

3.4.1 Repeated and Cyclic Treatment with CORT Resulted in Progressively Larger Changes in FST Behaviors

In cycle 1, the CORT rats exhibited more immobility and less swimming in the FST after 21 days of treatment, but not after 10 days of treatment, which is consistent with previous reports (Brotto et al., 2001; Mitra & Sapolsky, 2008), and with the results of our

Table 3-2. Correlations between immobility in the forced swim test (FST) and RE-1 silencing transcription factor (REST) immunoreactivity in different subfields of hippocampus: hilus, stratum lacunosum, CA1.SO and CA3.SO (stratum oriens), CA1.SR and CA3.SR (stratum radiatum), in cycle 1. Asterisks (*) denote significant correlations ($p < 0.05$, $N = 9$).

Pearson's r	Hilus	Stratum Lacunosum	CA1.SO	CA1.SR	CA3.SO	CA3.SR
Immobility	0.627	0.730*	0.679*	0.733*	0.412	0.576

Table 3-3. Correlations between immobility in the forced swim test (FST) and RE-1 silencing transcription factor (REST) immunoreactivity in different subfields of hippocampus: hilus, stratum lacunosum, CA1.SO and CA3.SO (stratum oriens), CA1.SR and CA3.SR (stratum radiatum), in cycle 3. Asterisks (*) denote significant correlations ($p < 0.05$, $N = 9$).

Pearson's r	Hilus	Stratum Lacunosum	CA1.SO	CA1.SR	CA3.SO	CA3.SR
Immobility	0.890*	0.594	0.339	0.532*	0.811*	0.174

first experiment (Chapter 2). After 21 days of injection-free recovery, a decrease in immobility and increases in swimming and latency to immobility were observed. Finally, during the re-exposure to CORT treatment in cycle 2, a significant increase in depression-like behavior was re-established after only ten days of CORT administration, indicating that only half of the initial CORT exposure (10 days) was sufficient to produce depression-like behaviors in CORT rats in cycle 2. Similarly, following the second recovery period, the third re-exposure to CORT resulted in a pronounced increase in immobility, and decreases in swimming and climbing after ten days of CORT injections. The re-establishment of the depression-like behavior following shorter periods of glucocorticoid exposure suggests that the rats may have become sensitized to the deleterious effects of CORT over successive cycles of exposure. These results support previous studies showing sensitization of depression-like behavior in adult rats exposed to one week of variable stress and re-exposed to 90 min of restraint stress (Zurita et al., 2000). Additionally, it has been shown that chronically-stressed animals subjected to novel acute stressors show a sensitized release of stress hormones (Konarska, Stewart, & McCarty, 1989; Pardon, Ma, & Morilak, 2003) and neurotransmitters (Nisenbaum, Zigmond, Sved, & Abercrombie, 1991).

Importantly, the vehicle rats again have exhibited gradual increases in immobility from treatment cycle 1 to treatment cycle 2, similar to the results observed in Experiment 1 (Chapter 2) of this dissertation. In Chapter 2, we hypothesized that repetitive behavioral testing or task learning could have accounted for the slow increase in immobility seen in control conditions. The results observed here go against that hypothesis, as the non-repeated FST method used in this experiment produced analogous results, suggesting that other factors could be confounding the results of the FST behavior in rats, including aging, body weight changes, mild stress from daily subcutaneous saline injections, or stress from isolated single-housing of rats. Interestingly, the time spent immobile plateaus from cycle 2 to cycle 3 in the

vehicle rats, indicating that the potential effects of confounding factors are diminished at later stages of the experiment. Given the results of these experiments, the interpretation of sensitization of CORT-induced effects in the FST should be made with caution. It is possible that the sensitization could be inferred from the behavior in cycle 3 only, considering that the immobility behavior of vehicle rats stabilizes at these time points. Similar to Experiment 1, we have created and analyzed ratio scores in the attempt to account for the potential effect of body weight increases on the FST behaviors. Again, the IBW ratio of the vehicle rats eliminated the nonspecific increase in immobility in these rats that was seen over the course of three cycles of testing, whereas the CORT rats exhibited the expected cycling in immobility behavior throughout treatment with and recovery from CORT exposure.

Data from this experiment further provide some support for recovery worsening in CORT-treated rats (observed in the original Experiment in Chapter 2) across three cycles of treatment (Table 3-1). Interestingly, CORT rats have showed not only a lower rate of recovery in cycle three compared to cycle two, but also their IBW ratio during recovery in cycle three was significantly different from the vehicle rats' IBW ratio. This finding indicates that IBW ratio of CORT rats no longer normalized to the levels of control rats after three cycles of treatment. The observed worsening of recovery may be analogous to the phenomenon of "partial remission" in treated depressed patients (Fava et al. 2007) and is considered an important contributor to the relapse of depression episodes (Moylan et al., 2012).

3.4.2. Possible Sensitization of the Effect of CORT on Sucrose Preference Across Cycles of Treatment

The effects of CORT on sucrose preference were different across cycles of exposure. Specifically, no significant changes in sucrose preference were observed in cycle 1 of CORT

treatment, however, after 21 days of the 2nd CORT re-exposure, the CORT rats showed a significant decrease in sucrose preference. Finally, after 21 days of the 3rd re-exposure to CORT, anhedonia-like behavior was observed again in CORT rats. These results suggest that CORT had greater effects on sucrose preference in cycles two and three than in cycle one, which again points to a possible sensitization of the depressogenic effects of CORT across cycles of exposure. Our findings of behavioral sensitization associated with stress re-exposure and recurrent depression-like episodes in rats are supported by a paper that reported similar, but more pronounced effects of stress on rodent sucrose consumption (Remus et al., 2013). Specifically, the researchers reported that when rats were exposed to 35 days of CMS and 20 days of recovery, and then were re-exposed to CMS for 15 days, they exhibited a more rapid decline in sucrose consumption compared with non-stressed control rats. Our results also suggest anhedonia sensitization, but at a later time point of stress re-exposure. Therefore, it appears that sensitization of stress can occur with other stress paradigms in addition to CORT administration, but the exact timing of the sensitization can vary depending on differences in experimental protocols such as length and severity of stress exposure and the type of behavioral testing that is employed.

3.4.3. CORT- and Vehicle-Treated Rats did not Differ in General Locomotor Behavior

We found no significant differences in OFT behavior between CORT or vehicle rats exposed to one, two or three cycles of treatment. This finding suggests that prolonged treatment with CORT does not alter nonspecific locomotor activity. These results are consistent with previous findings in our laboratory (Marks et al., 2009), which showed that rats treated with CORT for 3 weeks were not different from control rats in the OFT locomotor activity and muscle strength in the wire suspension test, suggesting that the effects of CORT on depression-like behavior were not accounted for by the general metabolic effect

produced by glucocorticoids. Low to moderate levels of CORT are known to have anabolic effects, however, at chronically high levels CORT has a catabolic effect on muscle tissue (Tempel & Leibowitz, 1994). In the present experiment, we found that the CORT rats showed a significant decrease in body weight gain after ten days of treatment, and continued to do so throughout the entire experiment. Therefore, it was important to confirm that potential catabolic effects of prolonged high CORT levels were not sufficient to affect general locomotor activity and cloud the interpretation of the FST behaviors. Studies from our laboratory as well as others have shown that CORT-treated rats exhibit similar muscle strength on the wire suspension test (Marks et al., 2009), swim the same distance as control rats in a Morris Water Maze (Sousa, Lukoyanov, Madeira, Almeida, & Paula-Barbosa, 2000) and engage in more lever pressing for electrical stimulation than control rats (Barr et al., 2000). Taken together, we can conclude that in the present study, repeated and cyclic CORT administration produced a significant change in the forced swimming behavior of rats, which is indicative of the depression-like phenotype rather than a reduction in general locomotion.

3.4.4. Repeated and Cyclic CORT Treatment Decreases the Number of DCX+ Cells and their Maturation Rate, in GCL and SGZ

The present experiment examined whether the accumulative effects of CORT on behavior are paralleled by significant changes in hippocampal neurogenesis. Reduced neurogenesis is hypothesized to be involved in the pathophysiology of major depression. This hypothesis is largely supported by preclinical studies using chronic stress paradigms (Barha, Brummelte, Lieblich, & Galea, 2011; Brummelte & Galea, 2010; Duman et al., 2001; Gould et al., 1997; Pham et al., 2003; Snyder, Soumier, Brewer, Pickel, & Cameron, 2011). Work from our laboratory has shown that repeated administration of CORT produces time-dependent (7, 14, and 21 days) and dose-dependent (5, 10, and 20 mg/kg) decreases in the

number of DCX+ cells and their morphology (Lussier et al., 2013; Lussier, Romy-Tallón, Kalynchuk, & Caruncho, 2011). In the present experiment, we discovered that repeated and cyclic CORT treatment exerted increasingly greater deleterious effects on the number and dendritic complexity of immature dentate granule cells over time. Specifically, we found a non-significant decrease in the number of DCX+ cells in the 1st cycle of CORT treatment, and significant decreases in the number of DCX+ cells at the end of the 2nd and 3rd CORT re-exposures. Previous findings in our laboratory (Lussier et al., 2013) have shown that 21 days of 40 mg/kg CORT significantly decreases the number of DCX+ cells in the GCL. Here, CORT clearly decreased the number of DCX+ cells, but this effect just missed the statistical significance. This is likely due to the fact that we used a lower dose of CORT (i.e., 20 mg/kg) than the one used in the previous experiment.

With the exception of the last recovery period, we did not find significant recovery (to the levels of control rats) of DCX cell populations after the cessation of CORT treatment, unlike the reversal of dendritic retraction of hippocampal neurons that had been reported previously (Conrad et al., 1999). This finding supports evidence from clinical studies demonstrating that repeated stress during recurrent episodes of major depression may result in growing hippocampal injury observed as total volume loss (Sheline et al., 1999). Our data are also consistent with reports from preclinical research showing the progressive impairment of proliferation and survival of new neurons in the rodent dentate gyrus following three and six weeks of restraint stress (Pham et al., 2003). In addition, elevated levels PSA-NCAM were reported after three weeks of stress and recovery, but not after six weeks of stress and recovery, which suggests that shorter durations of stress may induce adaptive structural plasticity, whereas prolonged chronic stress may result in more profound and permanent damage to the hippocampus (Pham et al., 2003). Finally, Sapolsky et al. (1985) also demonstrated that prolonged CORT injections (at the high physiological range) irreversibly

depletes glucocorticoid receptors and produces a significant loss of neurons expressing
CORT receptors in CA3 subfield of rat hippocampus, closely resembling the characteristics
of the aged hippocampus (Sapolsky, Krey, & McEwen, 1985).

Importantly, in the present experiment, we found that vehicle rats also exhibited
gradual decreases in the number of DCX+ cells over three cycles of injections. Neurogenesis
is known to decline dramatically throughout adulthood and normal aging in rodents (Kuhn,
Dickinson-Anson, & Gage, 1996) and humans (Manganas, Zhang, Li, & Hazel, 2007).
Although the oldest rats in the experiment were approximately 6.5 months old and considered
to be in the mature adulthood stage (Sengupta, 2013), in addition to potential aging effects it
is plausible to suggest that daily subcutaneous vehicle injections could be a mild chronic
stressor for control rats. This could explain the gradual decreases in DCX+ cell number and
gradual increases in the immobility of our control rats. This is an interesting topic for further
study because nonspecific alterations in behavior or neurobiological measures over time in
control rats creates difficulties for the types of longitudinal studies that are needed to fully
understand the deleterious effects of chronic stress.

We also found that DCX+ cells in CORT-treated rats were characterized by reduced
dendritic complexity compared to vehicle-treated rats. CORT produced significant effects on
neuronal maturation: CORT treatment was associated with more category two DCX cells (at
the proliferative stage of development) and fewer category five or six cells (cells with defined
dendritic branching). In general, a larger percentage of DCX+ cells from the CORT-injected
rats had no or minimal dendritic branching compared to the vehicle-injected rats. Similarly, a
smaller percentage of DCX+ cells from the CORT-injected rats had a well-developed
dendritic tree. These results suggest that in addition to the glucocorticoid-induced cell loss in
the hippocampus, the newborn cells that do survive show slower maturation that can impede
their integration into the existing circuitry, and contribute to the impairment of hippocampal

plasticity and function. Our data revealed that some changes (percent of category five and six cells) in neuronal maturation were observed in cycle 1 and maintained through cycle 3, while other changes (percent of category two cells) became statistically significant only in cycle 3. Collectively, our findings provide some support to the idea that an exacerbation of neuropathological changes accumulating over several episodes of stress/depression may be an important factor in mood cycling and susceptibility to relapse.

Finally, the analyses of correlations revealed that a larger number of DCX+ cells in the GCL and SGZ was associated with less time spent immobile in the FST. Furthermore, less time spent immobile was associated with a greater percentage of category six (more mature) DCX+ neurons and smaller percentage of category three (less mature) neurons. These findings further support the role of dysregulated neurogenesis in the development and expression of depression-like symptoms in rodents.

3.4.5. Bi-Directional Changes in REST Immunoreactivity and the Number of REST+ Cells in Hippocampus of Rats Treated with One or Three Cycles of CORT

There is growing evidence that abnormal transcriptional regulation plays a significant role in the pathogenesis of many neuropsychiatric disorders, including mood disorders (Duman & Monteggia, 2006; Tsankova et al., 2007). REST is a transcription factor that binds to the DNA chromatin at the RE1 and other motifs, promoting the repression of various target genes, which are largely expressed in neurons (Chong et al., 1995; Schoenherr & Anderson, 1995; Tapia-Ramírez et al., 1997). Many of those genes (e.g., CRH, BDNF, and 5-HT1A serotonin receptor) are altered in conditions of stress and depression (Cieślik et al., 2011; Francis, Diorio, Plotsky, & Meaney, 2002; Goodfellow et al., 2009; Kolasa et al., 2014).

In the present study, we examined how REST protein levels were altered depending on the duration of CORT treatment. In the 1st cycle of treatment, we found that the number of

REST+ cells was upregulated in CORT-treated rats in several hippocampal subregions (i.e. CA1 and CA3 strata radiatum) compared to vehicle-treated rats. Similarly, a recent study reported increases in depression-like behavior and mPFC mRNA REST4 levels (a splicing variant of REST) in rats subjected to early life maternal separation (Uchida et al., 2010). In addition, upregulation and downregulation of a number of target genes within the RE1 site were also reported. Interestingly, overexpressing REST in the mPFC in neonatal mice resulted in their enhanced vulnerability to developing depression-like behavior and an enhanced expression of REST4 target genes. However, overexpressing mPFC REST in adult mice did not yield such results (Uchida et al., 2010). Additionally, a study by Mandel et al. (2011) reported that the sustained presence of REST (in utero overexpression) inhibited neuronal migration and differentiation, resulting in a subcortical band heterotopia-like phenotype, similar to the loss of DCX. Our correlation analyses also demonstrate that higher levels of REST in CA3 were associated with lower numbers of DCX+ cells in the GCL and SGZ in rats exposed to one cycle of CORT. In addition, lower levels of DCX+ cells and higher levels of REST were strongly associated with higher immobility scores of rats in the FST.

Intriguingly, the repressive activity of REST is accompanied by a recruitment of histone deacetylases and chromatin-remodelling enzymes, such as methyl CpG binding protein 2 (MeCP2) (Ballas et al., 2005), which is also known to be altered in major depression (Schroeder et al., 2007; Tsankova et al., 2007). For example, MeCP2-deficient hippocampal neurons have fewer dendritic spines and reduced dendritic branching, and exhibit impaired neuronal maturation (Smrt & Zhao, 2010; Zhou et al., 2006). Similarly, research in our laboratory showed that one cycle of CORT administration upregulated MeCP2 expression in the hippocampal SGZ (Fenton & Kalynchuk, unpublished data). Furthermore, growing evidence suggests that MeCP2 is directly associated with the

regulation of the reelin and BDNF genes (Martinowich et al., 2003; Matrisciano et al., 2013; Tsankova et al., 2006), both of which are decreased in depressed patients and in CORT-treated rats, and are required for normal neurogenesis and neuronal maturation (Fatemi, Earle, & McMenomy, 2000; Karege et al., 2002; Lussier et al., 2009; Teixeira et al., 2012; Lussier et al., 2013). Together, these findings suggest that activation of REST-mediated pathways after the first exposure to CORT could be responsible for the generation of depression-like behaviors and dysregulated hippocampal function in rodents.

In contrast, after three cycles of treatment, the CORT rats exhibited significant decreases in both the number of REST⁺ cells (i.e., in hilus, stratum lacunosum, CA1 stratum radiatum, CA3 stratum oriens) and the density of REST protein (i.e., in GCL and CA3 pyramidal layers) relative to the vehicle rats. Our results demonstrate that CORT exerts bi-directional changes in REST expression depending on the duration of the exposure. Our observation that REST is decreased after three cycles of CORT exposure is supported by a clinical study showing that patients with major depression and bipolar disorder have significant decreases in REST mRNA in peripheral blood cells while experiencing a depressive mood, but not during remission (Otsuki et al., 2010). Unfortunately, no information was provided on the number of depressive episodes experienced by the patients who participated in the study, therefore, no conclusions about the time-dependent changes of peripheral REST mRNA can be drawn.

REST expression is sensitive to aging and age-related pathologies. Interestingly, a loss of REST in hippocampal and PFC neurons was found to be closely associated with Alzheimer's pathology, increased pro-apoptotic gene expression and oxidative stress (Lu et al., 2014). And conversely, an increase of REST expression in these neurons during healthy aging was correlated to cognitive preservation and longevity in humans (Lu et al., 2014). Taking into consideration the high incidence of Alzheimer's disease in depressed individuals

(Olin, Katz, Meyers, Schneider, & Lebowitz, 2002; Saczynski et al., 2010, for review see Diniz, Butters, Albert, Dew, & Reynolds, 2013), alterations in REST-mediated pathways could be a common underlying pathology of these comorbid disorders. Our results are consistent with the hypothesis that REST exerts neuroprotective effects in healthy aging. The control rats in our experiment showed significant increases in REST optical density from cycle 1 to cycle 3, whereas the CORT rats generally had either no change or less REST after cycle 3 than after cycle 1. The CORT-induced deficiency in REST immunoreactivity in GCL and CA3 and REST+ cells in several subregions of hippocampus could indicate a loss of neuroprotection as a result of multiple bouts of CORT exposure and aging. Additionally, our data indicates that in rats treated for three cycles, lower hippocampal REST expression was associated with fewer DCX+ neurons, a smaller percentage of category five (mature) dentate granule cells, greater immobility in the FST, and decreased sucrose preference. Finally, the important role of REST in neurogenesis was highlighted by a study reporting that REST impacts adult neurogenesis by restricting the neurogenic program in quiescent stem cells (Gao et al., 2011). Gao and colleagues showed that conditional knockout of REST from neural stem cells in mice leads to a transient increase in neurogenesis and, ultimately, to a depletion of the neural stem cell pool and fewer granule neurons. Taken together, these data suggest that altered REST protein expression in the hippocampus could be part of the mechanism by which CORT increases depression-like behavior and decreases adult hippocampal neurogenesis.

In summary, the results of this experiment indicate that repeated and cyclic exposure to high levels of CORT results in increases in depression-like behaviors, including higher immobility and lower sucrose preference scores, but not in general locomotor behaviors. These behavioral changes are paralleled by decreases in neurogenesis, specifically the number and maturation rates of DCX+ immature neurons in the GCL and SGZ. Furthermore,

a precise and time-dependent regulation of REST levels in the hippocampus could play an important role in the initiation and progression of depression-like symptoms in rodents. Specifically, after the first exposure to CORT, increased REST expression could be a mechanism by which glucocorticoids facilitate alterations in the RE1-containing gene network that regulates adaptive cellular processes in the hippocampus. In contrast, the significant decrease in hippocampal REST levels after three cycles of CORT exposure could be a result of a dysregulated gene network mediating cell death and stress resistance (Lu et al., 2014). However, we did not test this assumption directly and, therefore, cannot make definitive conclusions about the affected genes that may be targeted by varying levels of REST expression in hippocampus.

CHAPTER 4

Cyclical Exposure to CORT in an Animal Model of Recurrent Depression Results in Alterations of Hippocampal Microglial Cells

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Abstract

Neuroinflammation has been associated with the development of a number neurodegenerative diseases and neuropsychiatric conditions, such as major depression. Microglia are resident brain immune cells that are known to exert their effects on multiple central nervous system functions, including neurogenesis and neuroinflammation. The current study sought to examine the influence of repeated and cyclic CORT administration on microglia cell morphology and activation in the rat hippocampus. Rats were treated with CORT injections or vehicle injections once per day for one, two, or three cycles (21 days each), each followed by a recovery period. Following treatment, the rats were sacrificed, and brain tissue was collected for immunohistochemical analyses of hippocampal microglia. We found significant decreases in microglial area fraction in GCL and hilus of the hippocampus in CORT rats treated for one and three cycles. Furthermore, microglial cell phenotype was found to be changed (decreased in the number of primary processes and ramifications) in the hilus of CORT rats treated for one cycle compared to vehicle rats, however, these decreases became statistically significant only in cycle three. These data indicate that glucocorticoids could have a cumulative effect on brain immune cell activation. In addition, these measures of microglial activation were strongly correlated with the higher immobility scores in the FST and deficient granule neuron maturation reported in previous chapters of this dissertation, suggesting that microglial activation may be associated with paralleled decreases in neurogenesis and slowed immature neuron maturation. These results are consistent with the hypothesis that neuroinflammation plays a role in depression and suggests that specific neuroinflammation mediated by activated microglia could represent a common pathway of depressive symptom recurrence in major depressive disorder.

4.1. Introduction

Growing data implicate immunological factors in the etiology and pathophysiology of depression, however, the mechanisms by which this occurs are unclear. Clinical studies report that individuals with major depression have increased cytokine levels in blood plasma and cerebrospinal fluid, which occur in the absence of the physical illness or infection (Alesci et al., 2005; Dahl et al., 2014; Dantzer et al., 2008; Kern et al., 2014). Meta-analysis studies also support the role of inflammation in depression by demonstrating consistent increases in TNF-alpha and IL-6 in plasma of depressed patients (Dowlati et al., 2010; Howren et al., 2009), and a reduction of IL-1 and IL-6 along with depressive symptoms in response to antidepressant treatment (Hannestad et al., 2011). In addition, plasma IL-6 levels were found positively correlated with several depressive symptoms, such as guilt and suicidal ideation (Alesci et al., 2005; O'Donovan et al., 2013).

Similarly, experimental research corroborates the clinical observations and reports elevated levels of cytokines in both plasma and brain tissue of animals with a depression-like phenotype (Golovatscka et al., 2012; Grippo et al., 2005; Yirmiya & Goshen, 2011; You et al., 2011). It has been demonstrated that acute and chronic stress, as well as glucocorticoid treatment, can increase cytokine mRNA and protein levels in the brain and leukocyte counts in plasma (Blandino et al., 2009; Bowers et al., 2008; Deak et al., 2005; Porterfield et al., 2012; Sorrells et al., 2009). Systemic and central treatment with pro-inflammatory cytokines (IL-1 or TNF-alpha) induce sickness behavior and depression-like behaviors in a dose- and time-dependent manner (Dantzer, 2004; Dunn & Swiergiel, 2005; Laugeray et al., 2011). Likewise, patients treated with immunotherapy (IL-2 and IFN- α) display signs of severe neuropsychiatric changes, including major depressive disorders (Denicoff et al., 1987; Renault et al., 1987).

Microglia are brain immune cells and a major source of centrally released cytokines (Walker et al., 2013a). Microglia have a dynamic morphology that is closely associated with their function. Under basal conditions, microglia are quiescent (“resting”), constitutively express macrophage proteins CD11b and Iba-1, and have very motile ramifications for continuous surveillance of their territory and communication with surrounding cells (Davalos et al., 2005; Hanisch & Kettenmann, 2007; Kettenmann et al., 2011; Nimmerjahn et al., 2005). However, under pathological conditions (e.g. neuronal damage), microglia can rapidly acquire a reactive profile, upregulate expression of antigen presentation molecules (CD80, CD86, CD40), generate reactive oxygen and nitrogen species, and release pro-inflammatory cytokines (Davalos et al., 2005; Johnson et al., 2002). In a reactive state, microglia are characterized by significant morphological transformations including a swollen cell body, thickened and shortened ramifications, and an amoeboid shape (Kettenmann et al., 2011).

The hippocampus is one of the brain regions most densely populated with microglial cells (Lawson et al., 1990). Interestingly, there is evidence that microglia can play an important role in neurogenesis by producing a number of growth factors, such as IGF-1 and BDNF (Butovsky et al., 2006; Coull et al., 2005; Sierra et al., 2010; Zhang et al., 2014) that play important roles in regulating neurogenesis. Moreover, microglia make frequent brief contacts with neurons at presynaptic boutons, postsynaptic spines, and the synaptic cleft, and therefore may contribute to the organization of neural circuits (Paolicelli et al., 2011; Tremblay et al., 2010; Wake et al., 2009).

Currently, it is not known if microglial reactivity is exacerbated by multiple cycles of CORT administration. Here we examined microglia activation in our animal model of recurrent depression and compared changes in microglia reactivity following one vs. three cycles of CORT treatment. We hypothesized a progressive, time-dependent increase in microglia activation, and that this activation would correlate with decreases in hippocampal

neurogenesis (number of DCX+ neurons). Furthermore, we expect that dynamic changes in microglial activation would be associated with depression-like behavior. Our findings indicate that CORT induced alterations in hippocampal microglial area fraction and cell morphology (decreased number of primary processes and ramifications), which are typical of microglial activation. Furthermore, we found that these measures of microglial activation were strongly correlated with higher immobility scores and slower rates granule cell maturation in the GCL and SGZ.

4.2. Materials and Methods

A subset of animals ($n = 32$) from Experiment 2 (Chapter 3) was used in the current Experiment.

4.2.1. Subjects

Male Long-Evans rats ($N = 183$) were used in this study (Charles River Laboratories, Canada). The rats weighed 225-250 grams upon arrival to our animal holding facility. They were individually housed in standard polypropylene cages with water and food available ad libitum. The temperature of the colony room was maintained at 21 °C, and all experimental procedures were conducted during the light phase of the light/dark cycle (lights on at 8 a.m.). All experimental procedures were carried out according to a protocol approved by the University of Saskatchewan Committee on Animal Care and Supply.

4.2.2. Experimental Procedures

The rats were handled briefly once per day for seven days prior to the start of CORT injections. We then weight-matched the rats and randomly assigned them to one of the following two treatment groups: repeated and cyclic treatment with 20 mg/kg CORT ($N =$

91) or repeated and cyclic treatment with vehicle (N = 92). Both CORT and vehicle groups were divided into subgroups according to the duration of their treatment: (1) 10 days of cycle one, (2) 21 days of cycle one, (3) recovery of cycle one, (4) 10 days of cycle two, (5) 21 days of cycle two, (6) recovery of cycle two, (7) 10 days of cycle three, (8) 21 days of cycle three, and (9) recovery of cycle 3. Each subgroup comprised 20-21 rats (CORT and vehicle). A subset of rats from the CORT and vehicle groups treated for one cycle, and the CORT and vehicle groups treated for three cycles were used in this experiment.

The CORT or vehicle injections were administered once per day for three cycles. Each cycle comprised 21 consecutive days, followed by a 21-day injection-free recovery period. All injections were administered between 9:00 and 11:00 a.m. CORT (Steraloids) was suspended in 0.9% (w/v) physiological saline with 2% (v/v) Tween-80 (Sigma Aldrich) and given in a volume of 1 ml/kg.

4.2.3. Perfusions and Tissue Preparation

Rats were anesthetised with an overdose of intraperitoneal sodium pentobarbital and perfused transcardially with 0.1 M phosphate buffer (PB, pH 7.4) followed by 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed and postfixed in 4% (w/v) paraformaldehyde for 48 h at 4°C. Then the brain tissue was sectioned at 50 µm using a vibratome (Vibratome 3000, Vibratome Company, St. Louis, MO, USA) and stored at -20°C in a cryoprotectant solution with 30% (v/v) ethylene glycol, 1% (w/v) polyvinylpyrrolidone, and 30% (w/v) sucrose in 0.1 M PBS (pH 7.4).

4.2.4. Immunohistochemical Procedures and Analyses

A subset of rats from cycles one and three (CORT-cycle 1: n = 5-8; CORT-cycle 3: n = 5-8; vehicle-cycle 1: n = 5-8; vehicle-cycle 3: n = 5-8) was used for the

immunohistochemical analyses in the current experiment. Every 12th section of the brain (from approximately -2.40 mm to -3.94 mm from bregma) was used for immunostaining. All immunostaining procedures were done in 6-well tissue culture plates. Brain sections from each treatment group were counterbalanced across all 6-well culture plates to ensure consistency in immunohistochemical processing. Finally, to confirm the specificity of the antibody used in immunostaining, one well with brain sections served as control and was subjected to immunohistochemical procedures in the absence of the primary antibody. We did not observe any immunoreactivity in the absence of the primary antibody.

4.2.5. Immunohistochemical Procedures

To visualize microglial cells, CD11b immunohistochemistry was conducted. Briefly, free-floating tissue sections were rinsed in 0.1 M TBS (pH 7.4) and incubated in 1% H₂O₂ for 30 min to block endogenous peroxidase activity. Then the sections were blocked in a 0.3% (v/v) Triton X-100 TBS buffer containing 5% (v/v) normal horse serum (NHS) for 1 hour at RT, followed by incubation with a primary mouse anti-CD11b, clone OX-42, monoclonal antibody diluted in block solution (1:200; Millipore, USA) for 48 hours at RT. The tissue was then incubated with a biotinylated secondary antibody (horse anti-mouse; 1:200; Sigma-Aldrich) for 2 hours at RT, and then in an avidin-biotin complex (1:500, Vector Laboratories, USA) for 30 min at RT. Immunolabelling was visualized with 0.05% (w/v) DAB, 4.167% (w/v) nickel ammonium sulphate, and 0.0078% (v/v) H₂O₂ diluted in 0.175 M sodium acetate. After approximately 8 minutes, the reaction was stopped by rinsing the sections in 0.175 M sodium acetate. The sections were then mounted onto glass slides, dehydrated, and coverslipped using Entellan resin (Millipore, USA) for light microscopic examination.

4.2.6. Analyses of Immunohistochemical Staining

4.2.6.1. Quantitative Analysis of Microglial CD11b (OX-42) Structures in the Hippocampus

The areas occupied by CD11b microglial elements (area fractions) were measured per unit area of the tissue in the GCL, hilus, CA1 strata oriens and radiatum, and CA3 strata oriens and radiatum subfields of the hippocampus. This quantification was accomplished through Image J. In brief, monochrome images of hippocampal regions (three tissue sections per rat) were collected at 200X magnification using a Nikon E800 microscope. In Image J, a binary image was created through selecting a threshold value that kept all CD11b immunostained elements but no background. The ROI was traced and the area occupied by the CD11b structures (area fractions) within the ROI was then measured using the “Analyze Particles” command in Image J (Kodali et al., 2015).

4.2.6.2. Morphological Analyses of Microglia in Hilus

The morphology of randomly selected microglia cells in the hilus was examined using a neuron tracing system (Neurolucida; Microbrightfield) linked to a Nikon microscope. Hilus microglia were chosen based on the results of the microglial area fraction analyses and due to their greater accessibility for unobstructed tracing. Five microglia cells per rat (five rats per group) were randomly selected and individually traced in their entirety on the live image using a 100X lens with oil immersion. The following criteria were used for selection: (1) location of the cell body in the hilus; (2) full staining of the cell body and its processes; (3) most microglial processes unobscured by background staining or by other cells.

NeuroExplorer software (MicroBrightfield) was used for the quantification of somatic and process morphological parameters. Somatic metrics included cell body area and ferret maximum (the longest diameter of the soma). Total process length, the number of primary processes, the number of nodes and ends, and the average process diameter were quantified.

To compare branch architecture, a Sholl analysis was conducted in which the cell body was placed at the center of concentric circles (with diameters incrementing by 10 μm), and the number of intersections at all radial distances were quantified.

4.2.7. Statistical Analyses

Main effects and interactions were first analyzed using a two-way univariate ANOVA, with time and treatment as independent variables. Separate independent sample t-tests (one-tailed or two-tailed, as appropriate) were used to analyze treatment effect at specific experimental time points as per our a priori hypotheses. All data were analyzed using Statistical Package for the Social Sciences (Chicago, IL, version 18.0). The criterion for statistical significance of between-group differences was set at $p < 0.05$.

Bivariate Pearson's correlations were used to examine the statistical relation between measures of microglial activation, and the behavioral data and neurogenesis markers described in Chapter 3. The criterion for statistical significance of the correlational analyses was set at $p < 0.05$.

4.3. Results

4.3.1. Effect of Repeated and Cyclic CORT Treatment on Microglia Area Fraction

Measures

Figures 4-1 and 4-2 demonstrate the area fraction of CD11b microglial elements (soma and processes) in the hippocampus in CORT and vehicle rats in cycles one and three. The two-way ANOVA revealed significant main effects of time [$F(1, 28) = 24.612, p < 0.001$; $F(1, 28) = 15.568, p < 0.001$] and treatment [$F(1, 28) = 9.238, p = 0.005$; $F(1, 28) = 9.865, p = 0.004$] on microglia area fraction in the GCL and hilus. Similarly, there was a significant main effect of time [$F(1, 28) = 10.206, p = 0.003$; $F(1, 28) = 7.063, p = 0.013$] on

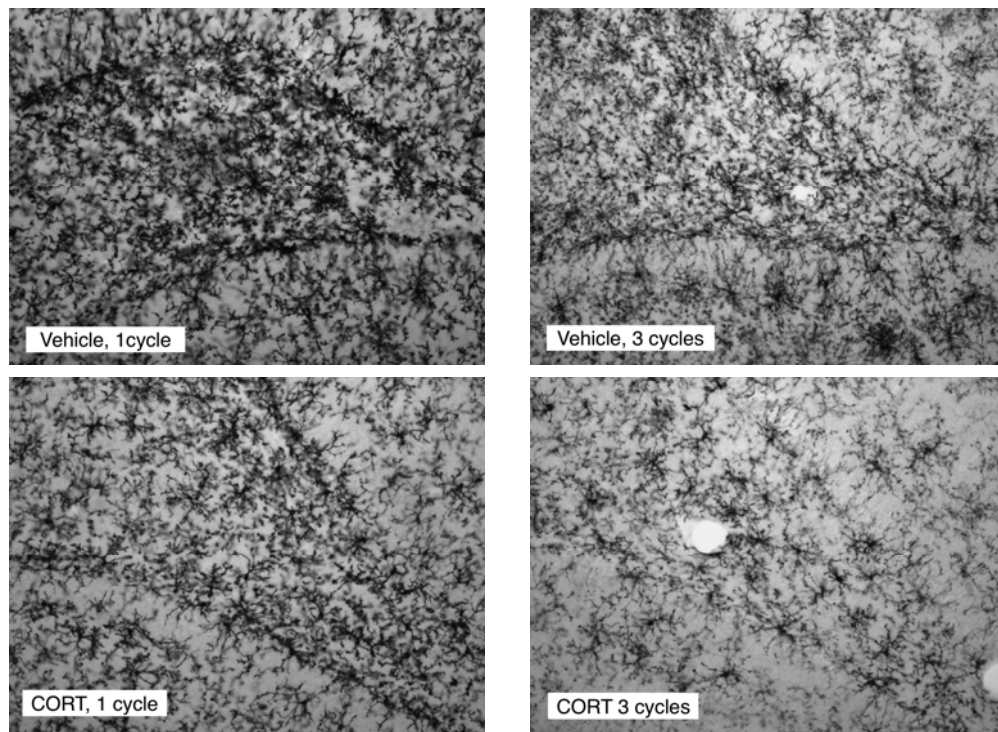
microglia area fraction in the CA1 stratum oriens and stratum radiatum, and a significant main effect of treatment on CA1 stratum oriens treatment [$F(1, 28) = 4.111, p = 0.05$], but not CA1 stratum radiatum (p values > 0.855). Finally, there was a significant main effect of time [$F(1, 28) = 5.33, p = 0.029$; $F(1, 28) = 5.94, p = 0.021$] on microglia area fraction in the CA3 stratum oriens and stratum radiatum, and a significant effect of treatment on CA3 stratum radiatum only [$F(1, 28) = 4.21, p = 0.05$]. There were no significant interactions of treatment by time on microglia area fraction of these hippocampal subregions (p values > 0.843).

In cycle 1, there was a significant decrease in area fraction in the hilus and GCL of CORT rats compared to the vehicle rats [$t(14) = 2.131, p = 0.025$, and $t(14) = 2.001, p = 0.033$, respectively]. Similarly, the CORT rats had a decrease in area fraction in other hippocampal subfields (i.e., CA1 strata oriens and radiatum, CA3 strata oriens and radiatum) but these differences did not reach statistical significance (all p values > 0.161).

In cycle 3, the CORT rats also had a decrease in area fraction of microglial elements in the hilus and GCL [$t(14) = 2.308, p = 0.019$, and $t(14) = 2.35, p = 0.019$, respectively]. And again, although the CORT rats had a decrease in area fraction in other hippocampal subfields (i.e., CA1 strata oriens and radiatum, CA3 strata oriens and radiatum), these differences were not statistically significant (all p values > 0.171).

Within the CORT groups, rats treated for three cycles of CORT had significantly lower microglial area fraction compared to rats treated for one cycle in GCL, hilus, CA1 stratum oriens and stratum radiatum, and CA3 stratum oriens and stratum radiatum [$t(14) = 4.43, p = 0.001$; $t(14) = 3.22, p = 0.003$, $t(14) = 3.005, p = 0.005$, $t(14) = 2.174, p = 0.023$; $t(14) = 1.886, p = 0.04$; $t(14) = 1.93, p = 0.036$, respectively]. Within the control groups, vehicle treated rats for three cycles showed significantly lower microglial area fraction

A



B

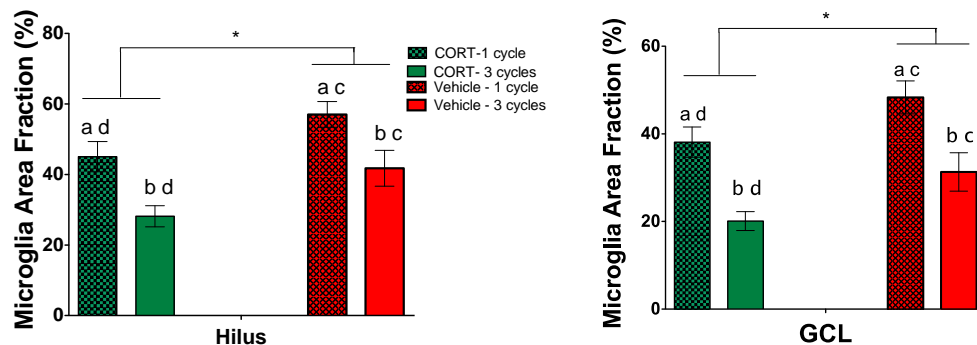


Figure 4-1. The effect of one and three cycles of repeated corticosterone (CORT) treatment on microglial area fraction measures in the granule cell layer (GCL) and hilar subregions of the dentate gyrus. Panel (A) shows representative photomicrographs of CD11b-positive microglia cells in the GCL and hilus of vehicle and CORT rats treated for one and three cycles. Panel (B) shows the quantification of area fraction of CD11b-positive structural elements in these brain regions in all four treatment groups. CORT produced significant reductions in area fraction of CD11b-positive microglia in both regions. Asterisks (*) indicate overall significant differences between treatment groups, respectively $p < 0.05$. Letters (a), and (b) represent significant differences between CORT and vehicle groups treated for one cycle and three cycles, respectively; letters (c) and (d) denote differences between two CORT groups and between two vehicle groups, respectively $p < 0.05$. Error bars denote the standard error of the mean (SEM).

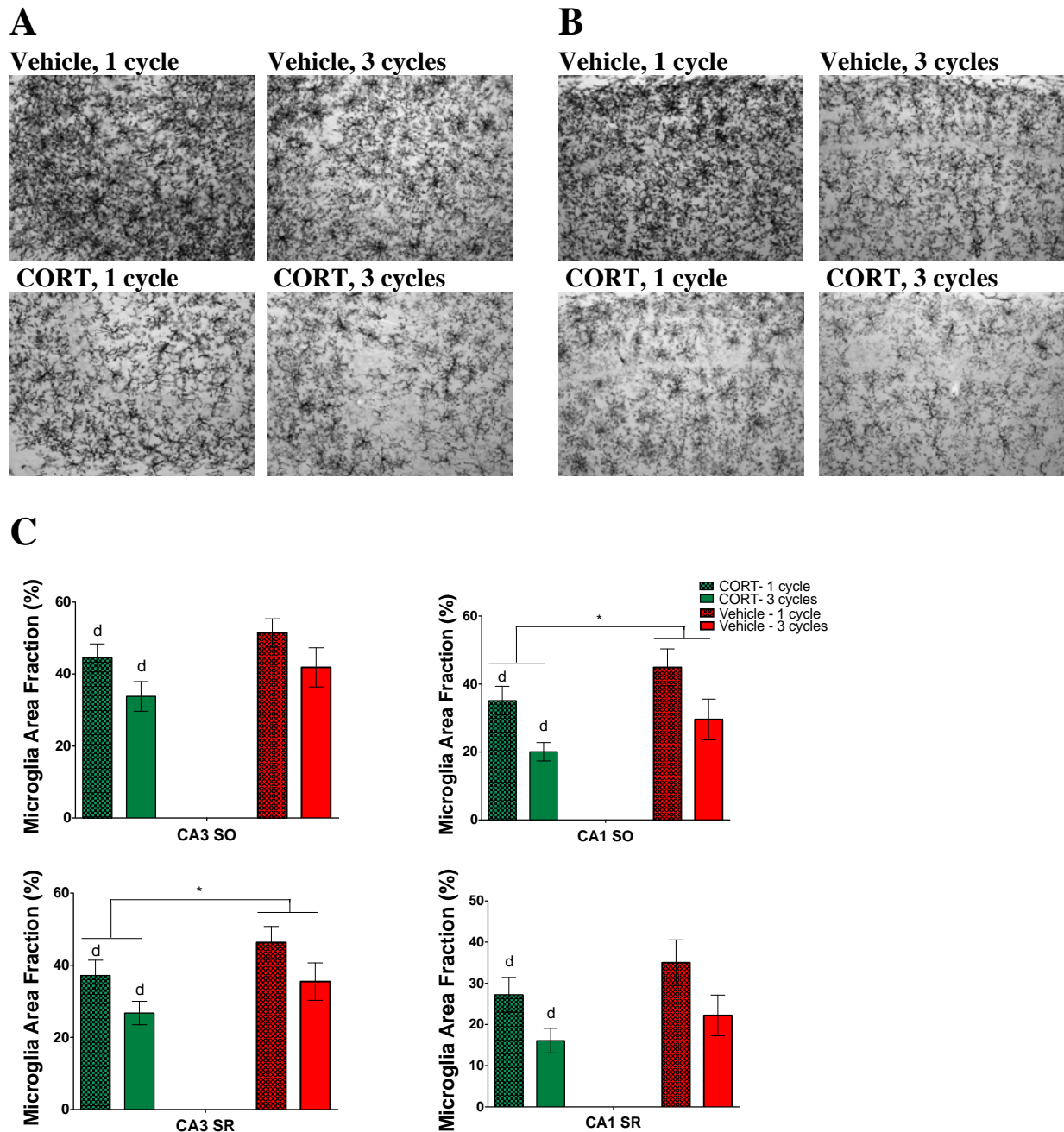


Figure 4-2. The effect of one and three cycles of repeated corticosterone (CORT) treatment on microglial area fraction measures in CA1 and CA3 hippocampal subregions. Panel (A) shows representative photomicrographs of CD11b-positive microglia cells in the CA3 subregion of the hippocampus in vehicle and CORT rats treated for one and three cycles. Panel (B) shows representative photomicrographs of CD11b-positive microglia cells in the CA1 subregion of the hippocampus in vehicle and CORT rats treated for one and three cycles. Panel (C) shows the quantification of area fraction of CD11b-positive structural elements in these regions in the four treatment groups. Asterisks (*) indicate overall significant differences between treatment groups, letter (d) denotes differences between two CORT groups $p < 0.05$. Error bars denote the standard error of the mean (SEM).

compared to rats treated for one cycle in GCL, and hilus [$t(14) = 2.95, p = 0.005$; $t(14) = 2.45, p = 0.014$].

4.3.2. Effects of Repeated and Cyclic CORT Treatment on Microglia Morphology

Figure 4-3 shows the results of the 3D reconstruction of individual microglia cells in the hilar region of the dentate gyrus in CORT and vehicle rats after cycles one and three. The two-way ANOVA revealed no significant main effects of time on microglial morphology measures: the soma area, total process length, the number of primary processes, the number of nodes or ends, the average process diameter, and the number of intersections (all p values > 0.273). However, there were significant main effects of treatment on the number of primary processes [$F(1, 16) = 7.491, p = 0.015$], the number of process nodes [$F(1, 16) = 5.142, p = 0.038$], the number of process ends [$F(1, 16) = 4.892, p = 0.042$], the total process length [$F(1, 16) = 5.537, p = 0.032$], the total number of process intersections [$F(1, 16) = 5.174, p = 0.037$], as well as the number of intersections at 20 and 30 μm distance from the soma [$F(1, 16) = 4.515, p = 0.05$; $F(1, 16) = 6.044, p = 0.026$]. There were no significant interactions of treatment by time on measures of microglial morphology (p values > 0.213).

In cycle 1, no statistically significant group differences were found in microglial soma area, total process length, the number of primary processes, the number of nodes or ends, the average process diameter, and the number of intersections between CORT and vehicle rats (all p values > 0.146).

In cycle 3, no statistically significant group differences were found in microglial soma area ($p = 0.277$). Compared to the CORT rats, the vehicle rats had significantly more primary processes [$t(8) = 2.2, p = 0.03$], a larger number of branching nodes [$t(8) = 2.13, p = 0.045$], a larger number of branch ends [$t(8) = 2.14, p = 0.045$], a larger total process length [$t(8) = 2.1$,

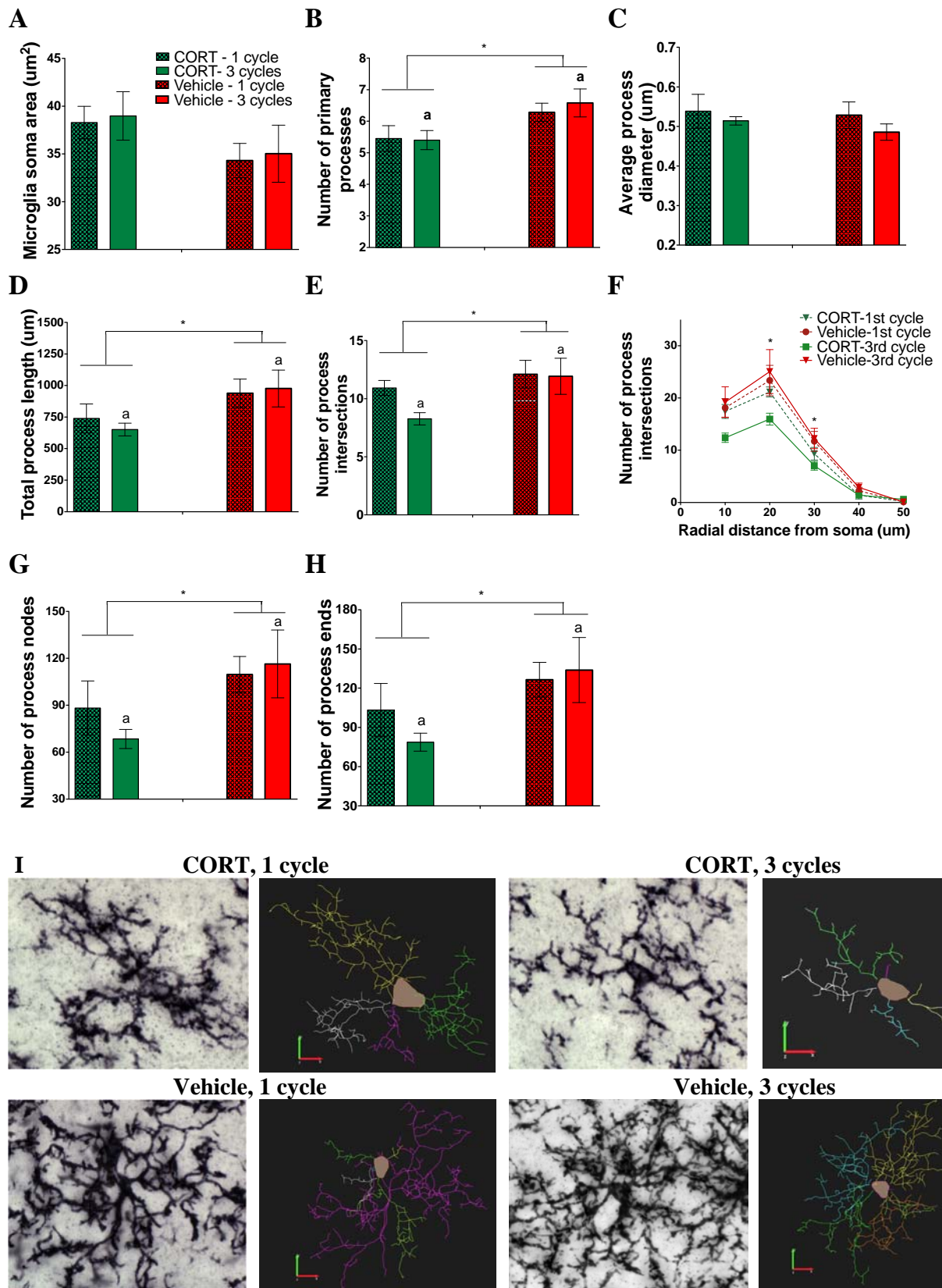


Figure 4-3. The effect of one and three cycles of repeated corticosterone (CORT) treatment on microglial cell morphology. Panel (A): quantification of microglia cell soma area; (B) quantification of microglial primary processes; (C): average microglial process diameter; (D) microglial total process length; (E) and (F): number of microglial process intersections in total and at different radial distances from soma; (G) and (H): numbers of microglial process nodes and ends; (I) representative micrographs of microglial cells in CORT and vehicle rats after one and three cycles, as well as a 3D reconstruction of one microglial cell created with Neurolucida software. CORT altered microglia cell morphology in cycle three but not cycle one. Microglia in CORT-treated rats displayed fewer and shorter processes, reduced ramification of processes (nodes and ends), and decreased number of intersections, implying a tendency towards conversion into activated or amoeboid microglia phenotype. No significant changes were observed in the average diameter of microglial processes, the size of the microglial cell body, or the soma size. Asterisks (*) indicate overall significant differences between treatment groups; letter (a) indicates significant differences between CORT and vehicle groups treated for three cycles, $p < 0.05$. Error bars denote the standard error of the mean (SEM).

$p = 0.034$], and a larger number of intersections [$t(8) = 2.23$, $p = 0.028$], specifically, at 10, 20, and 30 μm distance from the soma ($p = 0.04$, $p = 0.035$, and $p = 0.02$). No group differences were found in the average process diameter ($p = 0.252$).

4.3.3. Correlation analyses

Figure 4-4 illustrates selected correlations between measures of microglial activation, behavior, and neurogenesis in the CORT and vehicle rats from cycles one and three.

4.3.3.1. Correlations between Microglia Area Fraction and Behavioral Measures

Immobility scores were negatively correlated with microglia area fraction in the GCL (Figure 4-4A; $r = -0.498$, $p < 0.01$, $n = 32$), hilus (Figure 4-4B; $r = -0.458$, $p < 0.01$, $n = 32$), CA1 stratum oriens ($r = -0.405$, $p < 0.01$, $n = 32$), CA1 stratum radiatum ($r = -0.373$, $p < 0.05$, $n = 32$), CA3 stratum oriens ($r = -0.371$, $p < 0.05$, $n = 32$), and CA3 stratum radiatum ($r = -0.408$, $p < 0.05$, $n = 32$). Time spent swimming was correlated with microglia area fraction of the hilus ($r = 0.384$, $p < 0.05$, $n = 32$), CA1 stratum radiatum ($r = 0.392$, $p < 0.05$, $n = 32$), and CA3 stratum radiatum ($r = 0.360$, $p < 0.05$, $n = 32$).

4.3.3.2. Correlations between Microglia Area Fraction and DCX+ Cell Maturation Measures

The GCL microglia area fraction was negatively correlated with the percentage of DCX+ category two cells (Figure 4-4E; $r = -0.588$, $p < 0.05$, $n = 17$), and positively correlated with the percentage of DCX+ category five (Figure 4-4F) and category six cells ($r = 0.643$ and $r = 0.611$, respectively, $p < 0.01$, $n = 17$).

The hilus microglia area fraction was negatively correlated with the percentage of

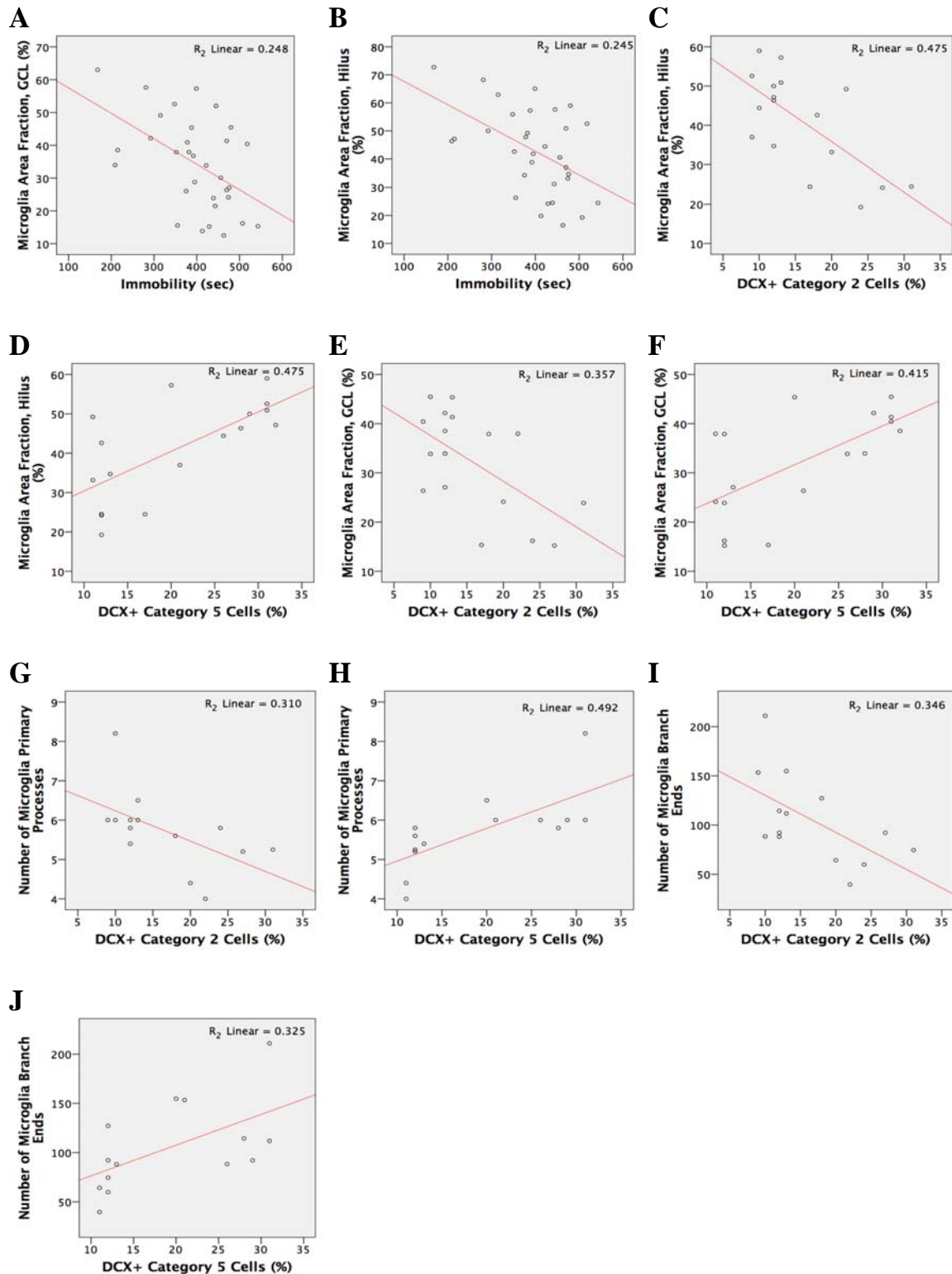


Figure 4-4. Correlations between measures of microglial activation and morphology, behavior, and neurogenesis in the corticosterone (CORT) and vehicle rats in cycles one and three. Correlation of immobility behavior with microglia area fraction in the granule cell layer (GCL, $r = -0.498$, $p < 0.01$) (A) and hilus ($r = -0.458$, $p < 0.01$) (B). Correlation of

microglia area fraction in the hilus with the percentage of DCX+ category two cells ($r = -0.681, p < 0.05$) (C) and category five cells ($r = 0.690, p < 0.01$) (D). Correlation of microglia area fraction in the GCL with the percentage of DCX-positive category two cells ($r = -0.588, p < 0.05$) (E) and category five cells ($r = 0.643, p < 0.01$) (F). Correlation of the number of microglial primary processes with the percentage of DCX-positive category two cells ($r = -0.542, p < 0.05$) (G) and category five cells ($r = 0.708, p < 0.01$) (H). Correlation of the number of microglial branch ends with the percentage of DCX-positive category two cells ($r = -0.574, p < 0.05$) (I) and category five cells ($r = 0.577, p < 0.05$) (J). For details, see the text.

DCX+ category two cells (Figure 4-4C; $r = -0.681$, $p < 0.05$, $n = 17$) and positively correlated with the percentage of DCX+ category five and category six cells (Figure 4-4D; $r = 0.690$ and $r = 0.640$, respectively, $p < 0.01$, $n = 17$). The CA1 SO microglia area fraction was negatively correlated with the percentage of DCX+ category three cells ($r = -0.511$, $p < 0.05$, $n = 17$) and positively correlated with the percentage of DCX+ category five and category six cells ($r = 0.506$ and $r = 0.602$, respectively, $p < 0.05$, $n = 17$).

The CA1 SR microglia area fraction was negatively correlated with the percentage of DCX+ category three cells ($r = -0.484$, $p < 0.05$, $n = 17$) and positively correlated with the percentage of DCX+ category six cells ($r = 0.540$, $p < 0.05$, $n = 17$).

The percentage of DCX+ category two cells was inversely correlated with the CA3 SR microglia area fraction ($r = -0.504$, $p < 0.05$, $n = 17$).

4.3.3.3. Correlations between Microglial Cell Morphology and DCX+ Cell Maturation Measures

The number of microglial primary processes was inversely correlated with the percentage of DCX+ category two (Figure 4-4G) and category four cells ($r = -0.542$ and $r = -0.599$, respectively, $p < 0.05$, $n = 14$) and positively correlated with the percentage of DCX+ category five cells (Figure 4-4H; $r = 0.708$, $p < 0.01$, $n = 14$). The number of microglial branch nodes was negatively correlated with the percentage of DCX+ category two and category four cells ($r = -0.576$ and $r = -0.551$, respectively, $p < 0.05$, $n = 14$) and positively correlated with the percentage of DCX+ category five cells ($r = 0.570$, $p < 0.05$, $n = 14$).

The number of microglial branch ends was negatively correlated with the percentage of DCX+ category two (Figure 4-4I) and DCX+ category four cells ($r = -0.574$ and $r = -0.568$, respectively, $p < 0.05$, $n = 14$) and positively correlated with the percentage of DCX+ category five cells (Figure 4-4J; $r = 0.577$, $p < 0.05$, $n = 14$).

4.4. Discussion

This experiment evaluated microglial activation following one and three cycles of repeated CORT treatment. Immunohistochemical characterization of CD11b (OX-42) suggested increased occurrence of reactive microglia exhibiting enlarged soma and fewer processes in CORT-treated rats, resulting in larger (white) spaces between microglial cells (Figure 4-1 and 4-2). In contrast, vehicle-treated rats displayed mostly ramified microglia (quiescent) with extensive fine branches. Using a thresholding technique, we quantified the area fraction of CD11b+ microglial elements (soma and processes) in several hippocampal subfields. This measurement revealed that after one cycle of treatment, there was an increased occurrence of ramified microglia (larger area fraction) in vehicle-treated rats compared to CORT-treated rats in the hilus and GCL regions of the dentate gyrus. Furthermore, after three cycles of treatment, this effect appeared to be potentiated, with CORT rats showing a smaller area fraction in the hilus and GCL, and therefore fewer microglial elements.

Although thresholding is a simple method, the interpretation of results using this method is not straightforward, as there is no direct relationship between the amount of elements measured (area fraction) and microglial activation. Therefore, the difference in thresholded material (area fraction) could represent a change in microglial cell number, a change in cell morphology, or both. To confirm the reactive state of microglia further, we traced a subset of CD11b+ microglia and their processes in the hilus from both vehicle and CORT-treated rats using Neurolucida (Figure 4-2). Most of the microglia in CORT rats treated for one cycle displayed shorter processes and reduced ramification of processes (decreases in the number of branching nodes and ends), reduced number of primary processes, and a reduced number of process intersections, but these effects became

statistically significant only after three cycles of treatment. The microglial soma size was enlarged in rats treated with one and three cycles of CORT, however this effect did not reach statistical significance. Consistent with area fraction analysis, the results of this morphological analysis suggest a gradual transition of microglia from a resting into an activated phenotype.

Overall, the findings from this experiment demonstrate evidence that repeated and cyclic treatment with CORT provoked significant alterations in the phenotype of hippocampal microglia through reductions in microglia cell ramifications. Microglial cell morphology is closely coupled with their functional properties (Stence, Waite, & Dailey, 2001; Streit, Walter, & Pennell, 1999). The observed morphological changes are consistently described in the literature (i.e., soma enlargement, retraction and thickening of extant branches) all of which are indicative of microglial activation (Beynon & Walker, 2012). Such activated microglia are known to release increased levels of pro-inflammatory molecules, including pro-inflammatory cytokines such as TNF- α , and free radicals such as nitric oxide (Liu & Hong, 2003; Tansey, McCoy, & Frank-Cannon, 2007). To my knowledge, we are the first to show that multiple exposures to CORT (three cycles) induce cumulative changes in microglial activation compared to one cycle of CORT, suggesting that repeated and cyclic glucocorticoid treatment has a neuroinflammatory effect in hippocampus. Intriguingly, REST protein is known to target the TNF superfamily of genes (Otsuki et al., 2010), and findings in Chapter 3 of this dissertation demonstrate significant decreases in hippocampal REST levels in rats treated for three cycles of CORT. These data further support the development of neuroinflammatory processes in these rats. Although our results are consistent with several studies reporting pro-inflammatory effects of chronic stress, it is important to note that they also contradict findings from another line of research. Glucocorticoids are traditionally known to have anti-inflammatory properties, because an

activated HPA axis can exert robust suppressing effects on the production of pro-inflammatory cytokines by inhibiting the activity of nuclear light chain kappa beta (NF- κ B), the key inflammatory transcription factor (Lee et al., 1988; Silverman & Sternberg, 2012; Zuckerman, Shellhaas, & Butler, 1989). Therefore, glucocorticoids have been reliably reported to reduce the expression of pro-inflammatory cytokines including IL-1, IL-6, and TNF- α and to increase anti-inflammatory mediators including IL-10 and TGF- β (Correale et al., 1998; Gayo et al., 1998). Moreover, the anti-inflammatory properties of glucocorticoids have been used pharmacologically to control post-surgical inflammation, as well as in cases of allergies, asthma, autoimmune diseases, etc. (Hockey et al., 2009). However, consistent with our results, several research studies showed that chronic stress resulted in an increase in microglia Iba-1 immunoreactivity and activation in several brain regions, including the hippocampus, PFC, and amygdala (Tynan et al., 2010a; Wohleb et al., 2011, 2012). In addition, high levels of CORT in rats exposed to unpredictable mild stress increased the levels of LPS-induced inflammatory signalling mediator NF- κ B in the hippocampus (Munhoz et al., 2006), whereas low concentrations of CORT inhibited the expression of pro-inflammatory cytokines mRNA IL-1 β and TNF- α in rat hippocampus following the kainic acid stimulation (MacPherson, Dinkel, & Sapolsky, 2005). Furthermore, in humans psychological stress was found to initiate an acute catecholamine response with the release of pro-inflammatory cytokines from mononuclear cells in the periphery (Bierhaus et al., 2003). The ability of CORT to exert both pro-inflammatory and anti-inflammatory effects on microglia has been elegantly investigated in a research study by Frank and colleagues (Frank et al., 2010). In their study, the authors showed that when repeated stress is administered prior to the immune challenge (LPS), the subsequent inflammation is potentiated. In contrast, when CORT was administered to microglia of

stressed rats 1 hour after the LPS challenge, the pro-inflammatory response was inhibited, suggesting a temporal relationship between the endocrine and immune systems.

As discussed earlier, major depressive disorder is frequently characterized by an elevation of both cortisol and pro-inflammatory cytokines, presenting a paradoxical overactivity of the immune and endocrine systems. Two theories have been proposed to resolve this paradox: one hypothesizes increased immunological activity as a result of developed glucocorticoid resistance, and the other theory highlights the evidence of both pro- and anti-inflammatory effects of glucocorticoids under specific conditions (Horowitz & Zunszain, 2015). Specifically, in the first theory, it is suggested that the initial exposure to CORT may desensitize or decrease the number of glucocorticoid receptors leading to a reduced intracellular glucocorticoid signalling that can no longer exert the same suppressive effects. This observation is supported by evidence from both clinical and preclinical literature demonstrating glucocorticoid resistance accompanied by high levels of pro-inflammatory cytokines in depressed patients (Bauer et al., 2003; Carvalho et al., 2008; Fitzgerald et al., 2006; Humphreys et al., 2006; Landmann et al., 1997; Maes et al., 1993) and in chronically stressed animals in models of depression (Avitsur et al., 2009; Sheridan et al., 2000). The second proposed theory is focused on evidence that glucocorticoids can be both pro- and anti-inflammatory under certain conditions (Frank et al., 2013; Sorrells et al., 2009). For example, animal studies demonstrated that exposure to glucocorticoids (or stress) after an inflammatory stimulus results in a suppression of cytokine release (Goujon et al., 1995). In contrast, exposure to glucocorticoids (or stress) before an inflammatory event heightens the inflammatory response (de Pablos et al., 2006; Espinosa-Oliva et al., 2011; Frank et al., 2010; Johnson et al., 2002; Munhoz et al., 2006, 2010). Similar results have been reported in preliminary studies in humans (Barber et al., 1993). Taken together, these data highlight the complex relationship between the stress and immune systems, suggesting that research

integrating both systems would help clarify the mechanisms involved in the etiology and pathogenesis of depression.

In addition to inducing neuroinflammation, microglia play an important role in adult neurogenesis. Several mechanisms have been proposed to explain how microglia can effect neurogenic function. For example, quiescent (ramified) microglia can produce a number of growth factors, such IGF-1 and BDNF, which promote cell proliferation (Butovsky et al., 2006; Coull et al., 2005; Sierra et al., 2010; Zhang et al., 2014). In addition, *in vitro* studies demonstrate that microglia have the ability to guide the differentiation of adult neural precursor cells toward a neuronal phenotype (Aarum, Sandberg, Haeberlein, & Persson, 2003). Furthermore, microglia were reported to make frequent brief contacts with neurons at presynaptic boutons, postsynaptic spines, and the synaptic cleft, and likely may contribute to the maintenance of neural circuits (Paolicelli et al., 2011; Tremblay et al., 2010; Wake et al., 2009). Finally, some of the well-known beneficial effects of environmental enrichment on neurogenesis may be mediated by microglia. Vukovic et al. (2012) demonstrated that when hippocampal microglia extracted from the runner mice were placed to the hippocampal neurosphere culture of sedentary mice, the number of NPCs in the culture of sedentary mice significantly increased, implying that microglia can be altered by enrichment to exert modulatory neurogenic effects. In fact, following an enriched experience, beneficial (ramified) microglia cell numbers increase, and this increase is accompanied by an increase in neurogenesis (Choi et al., 2008; Ziv et al., 2006). Interestingly, stem/progenitor cell proliferation was inversely correlated with the density of microglia expressing the Iba-1 marker, which is expressed by activated microglia only (Gebara, Sultan, Kocher-Braissant, & Toni, 2013). Findings described in the previous chapter of this dissertation provide evidence of the cumulative loss of DCX positive neurons from the GCL and SGZ in rats treated with CORT for one to three cycles. Here we demonstrate a transition of microglial phenotype

(from resting to activated) in the same rats, moreover, the measures of microglial area fraction in multiple hippocampal subregions were strongly and inversely correlated with the percentage of category two and three neurons (less mature cells), while at the same time positively correlated with the percentage of category five cells (more mature cells). Similarly, several measures of microglial morphology (number of primary processes, the number of branch nodes and ends) were also correlated negatively with the percentage of category two and four cells, and positively correlated with the percentage of category five cells. Finally, increased immobility behavior was associated with lower microglial area fraction (less ramification). Overall, these findings suggest that more activation (i.e. smaller area fraction, fewer processes, ends, and nodes) of microglia in the hilus, GCL, CA1 and CA3 is associated with increases in depression-like behavior and a slowed maturation rate of newborn dentate granule cells. Thus, it is possible that some of the anti-neurogenic effects of stress are regulated by microglial activation.

The present experiment was a preliminary examination of how repeated and cyclic glucocorticoid exposure can influence the microglial morphology and thus microglial activation in the rat hippocampus. The progressive shift in microglial cell morphology in the hilus with more cycles of CORT treatment suggests that stress exerts cumulative effects on brain immune cell activation. Changes in microglial morphology co-occur with cumulative decreases in the number of DCX-positive cells and could represent a shared pathway in development and recurrence of major depressive episodes. Future work with this model of depression should examine whether these cells become primed and release increasing amounts of cytokine content to confirm an exacerbation of neuroinflammation. In addition, it would be of interest to investigate whether the observed microglial activation is resolved during the stress-free recovery periods.

CHAPTER 5

General Discussion

5.1. Summary of the Main Findings

The primary purpose of this dissertation was to develop an animal model of recurrent depression and to further advance our understanding of the neurobiological alterations associated with stress re-exposure and depression relapse. To accomplish this, I examined the effects of repeated and cyclic CORT injections on several measures of rat depression-like behaviors and associated changes in hippocampal neurogenesis, dendritic complexity, hippocampal REST protein expression levels, and hippocampal microglial activation.

In **Chapter 2**, I examined the effect of repeated and cyclic CORT injections for two complete cycles (21 days each) on rat behavior in the FST. The behavior of rats was measured in a repeated fashion, where all rats were tested in the middle and at the end of each CORT treatment cycle, and at the end of each recovery period. CORT produced increasingly greater effects on depression-like behavior. Specifically, after developing a depression-like phenotype following the first cycle of CORT treatment, rats displayed sensitized increases in the time spent immobile and decreases in the time spent swimming, following a brief (ten days) CORT re-exposure. Additionally, a progressive worsening of recovery of immobility behavior was observed with more exposures to CORT. However, the potential confounding effects of repetitive behavioral testing, body weight, and aging need to be considered in the interpretation of these effects. Therefore, to address the issue of repetitive behavioral testing, further refining of the animal model was required.

In **Chapter 3**, I examined the influence of repeated and cyclic CORT treatment on several measures of rat depression-like behavior, the number and maturation rate of immature dentate granule neurons, as well as changes in hippocampal REST protein expression. In this

experiment, all rats were naïve to behavioral testing, as all behavioral tests were conducted in distinct groups of rats. Consistent with results of the first experiment, CORT produced larger increases in depression-like behavior in cycles two and three, specifically, higher immobility, decreased swimming, and lower sucrose preference scores, but no changes in general locomotor or exploratory behaviors. Similar to Experiment 1, cyclic CORT treatment resulted in worsening of recovery of immobility behavior in cycle three. Exacerbation of depression-like behaviors was paralleled by the accumulated decline in neurogenesis and dendritic complexity of immature granule neurons in the GCL and SGZ. Finally, I found that CORT also exerted its deleterious effects on transcriptional regulation of hippocampal function, which was characterized by measuring REST protein levels within the hippocampus. The finding of precise bi-directional regulation of hippocampal REST depending on the duration of CORT exposure was particularly important, as it may contribute to the mechanisms of depressive symptom recurrence, raising the possibility that novel antidepressant drugs could target REST-related deficits.

Chapter 4 included an examination of the effects of repeated and cyclic CORT exposure on hippocampal microglia. The goal of this project was to gain preliminary insight into microglial activation and its potential role in the function and plasticity of hippocampus under conditions of repeated stress. CORT produced cumulative changes in hippocampal microglia area fraction and microglial cell morphology, which are typically associated with neuroinflammatory processes. Additionally, these changes were strongly correlated with decreases in dentate granule neuron maturation rate and increases in depression-like behavior. These results suggested to me that the progressive activation of hippocampal microglia may be associated with exacerbation of depressive symptoms and paralleled decreases in the complexity of hippocampal immature neurons. Although these findings are correlational, further analyses of the complex relationship between the endocrine and

immune systems may elucidate common mechanisms underlying up-regulated levels of neuroinflammation, overactivity of the HPA axis, and the disruption of hippocampal function in major depressive disorders.

The overall results of this dissertation demonstrate evidence that repeated and cyclic glucocorticoid treatment could be used as an animal model of recurrent depression, as it increasingly affects the severity of depression-like behavior and the quality of recovery in rats. The presented model provides stronger construct and face validities compared with other existing animal models of depression, and it provides an opportunity to investigate the neurobiological changes associated with depressive symptom relapse. Data presented in this dissertation suggest that accumulated decreases in neurogenesis and newborn cell dendritic complexity could be associated with increasingly severe depression-like symptoms. Furthermore, stress appears to have a critical influence on hippocampal REST levels and microglial phenotypes, both known to be highly involved in the regulation of hippocampal plasticity and function.

5.2. Repeated and Cyclic Glucocorticoid Exposure as an Animal Model of Recurrent Depression

Major depression is a chronic and highly recurrent disorder (Hollon et al., 2006; Kendler et al., 2000; Solomon et al., 2000). The recurrent aspect of this disorder, with relapses of depression episodes, significantly impact an individual patient and also put a strain on the public health system. Therefore, when we conduct research on depression, it is important to examine not only the onset of depression but also the mechanisms and risk factors of episode recurrence. The lack of efficacious antidepressant treatment specifically designed for preventing recurrent episodes is due, in part, to the absence of adequate animal models that recapitulate recurrent episodes of depression. As earlier discussed, to our

knowledge, only one study investigated the recovery of the depression-like behavior and a subsequent reinstatement of a depression-like episode (Remus et al., 2013). Although the authors demonstrated the sensitization of rat anhedonia-like behaviors following stress re-exposure, the neurobiological mechanisms of this sensitization, as well as other depression-like behaviors, were not studied.

The results from my dissertation show that repeated and cyclic glucocorticoid administration produces cumulative increases in depression-like behaviors (immobility and sucrose preference) and cumulative worsening of behavioral recovery quality with every subsequent glucocorticoid re-exposure. Moreover, the depressogenic effects of CORT were not accounted for by changes in nonspecific locomotor activity, as CORT had no significant effects on rat behavior in the OFT. Importantly, the depressogenic effects of CORT on behavior were replicated in two separate experiments with repeated and non-repeated behavioral testing, further increasing the validity of the model. The observed re-establishment of the depression-like behavior following shorter periods of glucocorticoid re-exposure is indicative of the potential sensitization to stress and depression-like episodes in CORT-treated rats. These results are consistent with previous research demonstrating sensitized depression-like behavior, as well as sensitized stress hormone release and neurotransmitter release, in rats exposed to the repeated stress and later re-exposed to an acute stressor (Konarska et al., 1989; Nisenbaum et al., 1991; Pardon et al., 2003; Zurita et al., 2000). Additionally, our finding of aggravation of anhedonia-like behaviors at the end of the 2nd and 3rd CORT exposures is supported by a study that reported similar effects of stress on sucrose consumption in rats exposed to chronic CMS and then re-exposed to the shorter course of the same CMS procedure (Remus et al., 2013).

Taken together, the overall findings of this dissertation and the literature suggest that repeated and cyclic treatment with stress or glucocorticoids produces greater depression-like

behavior in rats in response to smaller subsequent stress, reflective of a recurrent depression disease course. The proposed animal model provides stronger face and construct validities by recapitulating the depression disease course in addition to the core depressive symptoms (i.e. depressed mood and anhedonia). Importantly, this model can be used for researching the mechanisms of major depression recurrence, as well as investigating the potential therapeutic targets for preventing depression episode relapse.

5.3. Neurogenesis and REST in Depression

Decreased hippocampal neurogenesis has been implicated in the pathogenesis of depression since the 1990s. This hypothesis is widely supported by abundant preclinical evidence demonstrating that repeated stress (e.g., glucocorticoid administration, CMS, restraint stress, and others) inhibits the proliferation, differentiation, and survival of hippocampal neurons (Bambico & Belzung, 2013; Banasr & Duman, 2007). Clinical observations also indicate reduced hippocampal volumes in patients suffering from major depression (Sheline et al., 1996; Sheline et al., 2003). Further support for the neurogenesis hypothesis of depression comes from studies showing that antidepressant drugs increase the proliferation, maturation, and survival of new neurons within the hippocampus (Banasr & Duman, 2007). However, the precise nature of the relationship between neurogenesis and depression is far from being fully understood, as several lines of research report conflicting results. For example, post-mortem analyses of severely depressed patients' brains did not reveal major cell loss (Reif et al., 2006). Additionally, although some research identified neurogenesis as a critical process in antidepressant action (Santarelli et al., 2003), others have found that efficacy of antidepressant medications are independent of neurogenesis (David et al., 2009), suggesting a potential contribution of other factors to the depressive symptomatology. Finally, there are conflicting reports on what stages of neurogenesis are

most affected by stress: Some research demonstrates suppressed hippocampal cell proliferation following stress (Brummelte & Galea, 2010; Duman et al., 2001; Gould et al., 1997), whereas other studies reveal that stress majorly disrupts neuronal differentiation and maturation, but not proliferation (Mayatissa, Henningsen, West, & Wiborg, 2009; Jayatissa, Bisgaard, West, & Wiborg, 2008). Collectively, these results underline a complex association between neurogenesis, stress, depression, and antidepressants, and further suggest that studying this relationship in more longitudinal experimental paradigms (as such the type proposed in this dissertation) would allow for a more extensive investigation of the role of neurogenesis in mood disorders.

Adult neurogenesis is strictly controlled by various signalling pathways, neurotransmitter systems, and transcriptional regulatory processes (Ables et al., 2010; Lie et al., 2005; Mira et al., 2010; Deisseroth et al., 2004; Ge et al., 2006; Tozuka et al., 2005). Recent work has identified zinc finger protein REST as a master factor for both differentiating precursor cells and adult neurons as well (Baldelli & Meldolesi, 2015). Moreover, growing evidence implicates REST in various neurological and psychiatric conditions, including depression. However, its role in the pathogenesis of mood disorders remains unclear. A recent study reported that REST targets several genes that are known to be involved in stress and depression, including CRH, the 5-HT_{1A} receptor, BDNF, and the TNF superfamily (Otto et al., 2007; Otsuki et al., 2010). Additionally, REST was reported to regulate glucocorticoid synthesis (Somekawa et al., 2009), suggesting its important involvement in the stress response system. The current literature on the role of REST in depression revealed mixed reports. One study demonstrated increased expression of REST4 in the mPFC of mice subjected to an early life stress. Interestingly, REST4 overexpression in the mPFC of neonatal mice, but not adult mice, potentiated depression-like behaviors in response to repeated restraint stress (Uchida et al., 2010). In contrast, a clinical study on

patients with major depressive and bipolar disorders showed that REST mRNA levels were decreased in peripheral blood cells of patients in a current depressive, but not a remissive state (Otsuki et al., 2010). The reduction of REST mRNA was accompanied by increases in CRH, adenylate cyclase 5, and the TNF superfamily mRNA. Intriguingly, the results from Chapter 3 may elucidate some of the discrepancies between the opposing findings of the above-mentioned research studies. Specifically, the results from Chapter 3 demonstrate that changes in brain levels of REST are highly dependent on the number/duration of glucocorticoid exposure: Increases in the number of REST+ cells within the hippocampus were found in CORT-treated rats after the 1st cycle of CORT administration, whereas profound decreases in the number of REST+ cells within the hippocampus were found in CORT-treated rats after the 3rd CORT cycle. These results suggest that biphasic changes in hippocampal REST could reflect the cumulative neurobiological damage associated with the progression of the depressive disorder, as well as potential mechanisms of depression recurrence. Also, these findings emphasize the importance of identifying which neurobiological changes in depression could be transient and adaptational, and which ones could be long lasting, accumulative, irreversible and potentially crucial for subsequent depression relapse. Additionally, the findings from Chapter 3 indicate that decreases in hippocampal REST expression were associated with decreased neurogenesis and a slowed maturation rate of immature neurons in hippocampus, as well as increased depression-like behaviors, further supporting the role of REST in neurogenesis and depressive symptomatology.

Interestingly, similar decreases in hippocampal and PFC REST levels were revealed in the brains of patients with Alzheimer's disease, along with increased pro-apoptotic gene expression and oxidative stress. And conversely, the increase in REST expression was found in individuals experiencing healthy aging, cognitive preservation, and longevity (Lu et al.,

2014). Taking into consideration the high comorbidity between Alzheimer's disease and depression (Olin et al., 2002; Saczynski et al., 2010; Diniz et al., 2013), as well as consistent reports of impaired hippocampal neurogenesis in Alzheimer's genetic animal models (Demars, Hu, Gadadhar, & Lazarov, 2010; Donovan et al., 2006; Haughey et al., 2002; Zhang, McNeil, Dressler, & Siman, 2007), alterations in REST-mediated pathways could be a common underlying pathology of these both of these disorders. Collectively, these data strongly support a role for REST in the regulation of stress, neurogenesis, and the pathogenesis of mood disorders, therefore further investigation of REST in these disorders may allow for the development of novel targets for therapeutic intervention.

5.4. Role of Microglia in Depression

Mounting evidence suggests that chronic stress induces biological alterations in multiple interacting systems, particularly in the endocrine and immune systems, which result in an increased risk of disease, including major depression (Cohen, Janicki-Deverts, & Miller, 2007). For example, clinical and preclinical research consistently report elevated levels of pro-inflammatory cytokines in the plasma and cerebrospinal fluid of depressed individuals and the brains of animals exhibiting depression-like phenotypes (Alesci et al., 2005; Dahl et al., 2014; Dantzer et al., 2008; Kern et al., 2014; Golovatscka et al., 2012; Yirmiya & Goshen, 2011). Decreases in cytokine levels were found in response to antidepressant treatment (Hannestad et al., 2011). Furthermore, both animals and humans treated with cytokine therapies display signs of depressive symptomatology (Dantzer, 2004; Dunn & Swiergiel, 2005; Laugeray et al., 2011; Renault et al., 1987). Microglia are the primary source of centrally released cytokines (Walker et al., 2013a). These cells have a very dynamic morphology and function, and, under pathological conditions, can rapidly turn into a reactive state by altering the morphological characteristics (swollen soma, deramified

processes), upregulating the expression of antigen presentation molecules, generating reactive oxygen and nitrogen species, and releasing pro-inflammatory cytokines (Davalos et al., 2005; Johnson et al., 2002; Kettenmann et al., 2011). Therefore, studying changes in microglial reactivity may provide insight into the neuroinflammatory correlates of stress and depression.

Another potential implication for the microglial alterations in the brain is related to its effects on neurogenesis. For example, research has shown that pro-inflammatory cytokines produced by microglia impact neuronal functioning through apoptosis, disrupting synaptic plasticity, and neurogenesis (Hayley, Poulter, Merali, & Anisman, 2005; Michael Maes et al., 2009; McAfoose & Baune, 2009). For example, IL-1b and TNF-alpha have been shown to impair long-term potentiation (Cunningham, Murray, O'Neill, Lynch, & O'Connor, 1996), neural progenitor cell proliferation (Goshen et al., 2008; Koo & Duman, 2008; Kuzumaki et al., 2010), and induce neuronal apoptosis by enhancing glutamatergic activity and potentiating excitotoxicity (Ben-Hur et al., 2003; Zou & Crews, 2005). In contrast, resting (surveilling) microglia are known to play an important role in neurogenesis by producing a number of growth factors, such as IGF-1 and BDNF (Butovsky et al., 2006; Coull et al., 2005; Sierra et al., 2010; Zhang et al., 2014), facilitating synaptic pruning, and contributing to the organization of neural circuits (Paolicelli et al., 2011; Tremblay et al., 2010; Wake et al., 2009). Decreased hippocampal neurogenesis and a slowed maturation rate of newborn dentate granule cells were found after repeated and cyclic CORT exposure (Chapter 3), and these changes were accompanied by progressive changes in microglial morphology (i.e., deramification) (Chapter 4). Moreover, the depression-like symptoms of rats were found to be strongly associated with both disrupted neurogenic function and microglial activation (Chapters 3 and 4). Interestingly, neuroinflammation is frequently associated with normal aging and neurodegenerative diseases (Eikelenboom & Veerhuis, 1996), which are also characterized by increased basal cortisol levels (Csernansky et al., 2006; Davis et al., 1986;

Lupien et al., 1994; Rasmuson, Nasman, Carlstrom, & Olsson, 2002). Finally, the findings in Chapter 3 revealed substantial decreases in hippocampal levels of REST protein expression, which is known to target the TNF superfamily genes. Decreases in REST mRNA and increases in TNF-12,13 mRNA were reported in blood cells of depressed patients. These data further suggest the potentiation of neuroinflammatory processes in the brains of CORT-treated rats, which could be mediated through microglial activation and REST signaling within the hippocampus.

Taken together, research from this dissertation and other literature suggest that microglial activation could represent a putative mechanism by which glucocorticoids impair neurogenesis and the integration of new neurons into existing circuitry. Although microglial investigation in this dissertation provides preliminary correlational evidence that glucocorticoids may exert progressive effects on microglial activation in the hippocampus, more research is needed to determine how this process regulates neurogenesis and how it manifests in depression-like behaviors.

5.5. Limitations

5.5.1. Can Depression Be Modeled in Laboratory Rodents?

Creating animal models of affective disorders is an extremely challenging task for a number of reasons. First, many of the symptoms used for depression diagnosis (DSM-V) are uniquely human (e.g., suicidal ideations, guilt, sadness) and cannot be convincingly recapitulated in animals. Second, some features of the human depressive syndrome, such as despair and anhedonia, which have been replicated in animals, are still considered approximate correlates of the human disorder (Nestler & Hyman, 2010). Third, the diagnosis of depression is established on a set of heterogeneous and vaguely defined clinical symptoms (weight gain/loss, increased/decreased appetite, irritable/sad mood) in the absence of

objective biomarkers or neuroimaging tests, creating additional difficulties for both clinical and preclinical research.

Despite these difficulties, several animal models of depression have been developed, replicating separate endophenotypes of the depressive syndrome. The experimental chapters of this dissertation were focused on the exogenous CORT administration model, which shares a common criticism of many stress-induced models of depression – unrealistic emulation of stress. High doses of CORT injections (20-40 mg/kg) are needed to produce robust and reliable effects on rat depression-like symptoms in this model (Chapters 2 and 3, Johnson et al., 2006), however, these doses are supraphysiological, as they exceed the highest plasma concentrations of glucocorticoids (5 mg/kg) produced in response to acute stressors in rodents (Sandi, 1996). However, the CORT model allows for better control over glucocorticoid levels in experimental subjects, avoiding the typical habituation effects seen with physical stressors such as restraint (Galea et al., 1997; Gregus et al., 2005; Grissom et al., 2007). Additionally, it gives an opportunity to investigate the direct impact of stress hormones on brain and behavior. Interestingly, the use of unnatural stressors is a typical limitation of stress-induced models of depression, with exception of CMS and social defeat stress models. CMS is known to utilize the most realistic imitation of the daily stressors. However, this model also faces criticisms in that it is the least reproducible and may cause heterogeneity in response to the perceived stimuli in different animals (Willner, 1997). Finally, the social defeat model, despite having strong ethological and ecologic validities, is not feasible for use with female rodents, who do not readily exhibit aggressive behaviors (Hollis & Kabbaj, 2014).

This dissertation has taken the CORT administration model of depression a step further by incorporating the recurrent nature of major depression disorder. The improved face validity would allow for a more precise examination of the development of behavioral

vulnerability, as well as a better way to identify the mechanisms governing depressive episode relapse.

5.5.2. Sex Differences in Depression

No female subjects were used in the experiments of this dissertation. Therefore, there are some limitations in extrapolating the results of these experiments to the human condition. Epidemiological reports consistently demonstrate a higher prevalence of depression in females, and these differences are seen in various cultures, suggesting a biological basis (Angst et al., 2002; Grigoriadis & Robinson, 2007; Weissman et al., 1993). For example, there is evidence that female rodents have higher resting circulating CORT levels, as well as higher CORT levels following stress exposure, compared to males (Seale, Wood, Atkinson, Bate, et al., 2004; Seale, Wood, Atkinson, Harbuz, & Lightman, 2004), suggesting potential sex differences in HPA function. Furthermore, previous research has shown that the immune system shows a marked sex-based dimorphism, observed in differing susceptibilities of women and men to some immunological diseases (Da Silva & Hall, 1992; Miller & Hunt, 1996; Offner, Moore, & Biffl, 1999). Surprisingly, evidence from animal research investigating the behavioral and neurobiological effects of stress indicates that females are rather resistant to stress in preclinical models. For example, findings from our laboratory show that CORT administration for 3 weeks increases depression-like behavior in both male and female rats, but the effect was less pronounced in the females (Kalynchuk et al., 2004). Another study demonstrated that 20 days of CORT injections increased depression-like behaviors in the FST in males, but not in females (Hill et al., 2003). Similarly, CMS exerted more profound effects on depression-like behavior in male rats compared to females (Dalla et al., 2005; Kamper et al., 2009). Also, an acute stress was shown to enhance fear behaviors and learned helplessness in males, but to a lesser degree in females (Heinsbroek, Van Haaren,

Van de Poll, & Steenbergen, 1991; Steenbergen, Heinsbroek, van Hest, & van de Poll, 1990). Consistent with these behavioral observations, stress was shown to cause variable changes in the neurobiology of males and females. For instance, repeated CORT administration impairs neurogenesis in the dorsal and ventral hippocampus of male rats, and only in the ventral hippocampus of females (Brummelte & Galea, 2010). Additionally, repeated stress produced reversible atrophy of dendrites of pyramidal neurons in the hippocampus of males (Conrad et al., 1999), but this effect is not observed in female rats (Galea et al., 1997). Similar findings were demonstrated in a study comparing male and female primates exposed to repeated social stress (Uno, Tarara, Else, Suleman, & Sapolsky, 1989). Taken together, these data imply that female rodents, despite enhanced HPA axis activity, display a greater resilience to a wide variety of stress challenges.

Fluctuations in gonadal hormones across the estrous cycle present a potential explanation for female stress resistance. For example, during proestrus (characterized by high estrogen levels), female rats exhibit fewer anxiety-like behaviors and depression-like behaviors compared to male rats and females during diestrus (Frye & Walf, 2002). Similarly, in humans, women have greater risks of developing major depression during the postpartum period, when estradiol levels are low (Brummelte & Galea, 2010). In addition, higher estrogen levels have been associated with increased hippocampal cell proliferation and decreased cell death (Pawluski, Brummelte, Barha, Crozier, & Galea, 2009; Tanapat, Hastings, Reeves, & Gould, 1999).

Interestingly, the innate brain immune system may also play a significant role in the sexual dimorphism of depression prevalence. Although male rats have more microglia in the developing brain (Lenz, Nugent, Haliyur, & McCarthy, 2013; Schwarz, Sholar, & Bilbo, 2012), this difference is reversed in adulthood, when females have more microglia with activated morphology and greater levels of pro-inflammatory cytokines (Schwarz et al.,

2012). Estrogen may exert anti-inflammatory effects in the brain (Członkowska, Ciesielska, Gromadzka, & Kurkowska-Jastrzebska, 2006), therefore the correlation between low estrogen levels in females and increased risk of mood disorders could be mediated by alterations in microglia and neuroinflammation. Collectively, these data indicate that the complex relationship between sex, stress, the immune system and mood is critical and should be studied further, especially in research using models of diseases with high female prevalence.

5.6. Future Directions

5.6.1. How Can the CORT Model of Recurrent Depression be Further Improved?

As discussed in the introduction of this dissertation, it is hypothesized that individual sensitivity to stress grows and the stress threshold decreases with every subsequent episode of depression, leaving the depressed individual vulnerable to depression episode relapse following minor stressors (Post, 1985). Several lines of research support this hypothesis. For example, a relapse into a depressive episode has been correlated with minor, but not major life stressors (Kendler et al., 1999; Ormel et al., 2001). The preclinical literature also demonstrates that animals pre-exposed to stress exhibit a sensitized release of catecholamines and CORT following stress re-exposure (Gresch, Sved, Zigmond, & Finlay, 1994; Grippo, Cushing, & Carter, 2007). In addition, findings from developmental studies also revealed that animals stressed *in utero* or during adolescence display depression-like behaviors in adulthood (Morley-Fletcher et al., 2003; Zurita et al., 2000). Consistently, findings in this dissertation also demonstrate behavioral sensitization of rats exposed to the second and third cycles of CORT administration. Importantly, after 21 days of CORT injections and 21 days of CORT-free recovery, only half of the initial CORT exposure (i.e., ten days) was needed to produce increases in depression-like behavior. Therefore, it would be interesting to

investigate the progression of behavioral vulnerability in rats by reducing the number of CORT injections further. For example, in the 3rd cycle of CORT exposure, rats could be injected for five days only, and, in the 4th cycle, rats could be given a single dose of CORT or a mild stressor (restraint, noise, etc.). Such an experimental protocol would allow for a closer recapitulation of the human condition and a more precise evaluation of the mechanisms underlying stress sensitivity and vulnerability and related depressive episode recurrence.

Additionally, the current model could be further improved by including an additional control group to the experimental design. In this group, the rats would not be subjected to the initial cycle of CORT injections, but receive vehicle for 21 days. However, after the recovery period, this group would be given CORT for the first time, while the other groups will be exposed to CORT for the second time. The addition of this “control + stress” group would allow for stricter control over several confounding factors in the experiment, including the effects of aging and weight gain.

5.6.2. Other Brain Structures that may be Involved in the Pathogenesis of Depression

The hippocampus is highly stress-sensitive, and therefore, it has been one of the most extensively studied brain regions in depressed patients and preclinical models of depression (Sterner & Kalynchuk, 2010). However, there are several other brain structures that are known to play important roles in emotion, mood, and stress, and these regions have also been implicated in depressive disorders. For example, decreases in PFC volume have been observed in individuals with multiple episodes of depression (Bora, Fornito, Pantelis, 2012; Hastings, Parsey, Oquendo, Arango, & Mann, 2004) and in stress-induced animal models of depression (Akana, Chu, Soriano, & Dallman, 2001; Cerqueira et al., 2005; Radley et al., 2005; Wellman, 2001), which were correlated with impairments in cognitive performance on PFC-dependent tasks (Cerqueira et al., 2005). There is evidence that the PFC is abundant in

glucocorticoid and mineralocorticoid receptors, and therefore, that it contributes to negative feedback control of HPA axis activity (Diorio & Viau, 1993). Interestingly, altered prefrontal activity in depressed patients was predictive of a relapse into a subsequent episode of depression (Farb, Anderson, Bloch, & Segal, 2011), making this brain structure particularly interesting in studying the mechanisms of depression recurrence. Furthermore, alterations in REST protein expression were found in both hippocampus and PFC (Lu et al., 2014; Uchida et al., 2010), which were associated with vulnerability to developing depressive phenotypes in rodents (Uchida et al., 2010). Finally, more research demonstrates stress-induced structural remodeling of microglia in the PFC, suggesting increased levels of neuroinflammation (Hinwood, Morandini, Day, & Walker, 2012; Hinwood et al., 2013; Kopp, Wick, & Herman, 2013; Tynan et al., 2010b). However, it is still not known how changes in the PFC microglia progress over several cycles of repeated stress, and whether such changes correlate with the worsening of depressive symptoms. Therefore, examining how microglia function is altered in the PFC is important for further substantiating its role in the pathophysiology of depressive disorders.

The amygdala is another limbic structure that is potentially involved in the pathogenesis of major depression. Volumetric alterations and hyperactivation within the amygdala have been reported in individuals suffering from depression (Drevets, Price, & Furey, 2008; Drevets, 1999; Hastings et al., 2004; Sheline, Gado, & Price, 1998). Preclinical research demonstrated that chronic stress results in amygdaloid neuronal hypertrophy and hyperactivity, accompanied by potentiated anxiety and fear behaviors (Mitra, Jadhav, McEwen, Vyas, & Chattarji, 2005; Rosenkranz, Venheim, & Padival, 2010). Several studies revealed that chronic stress also induced changes in the activation of amygdaloid microglia (Wohleb et al., 2011), whereas minocycline, a microglial activation inhibitor, produced antidepressant effects accompanied by increases in monoamine levels in the amygdala

(Arakawa et al., 2012). These observations suggest that the regulation of microglial activation in amygdala could be important in antidepressant efficacy. Finally, investigations of REST protein expression in the amygdala have been very sparse. To my knowledge, only one study looked at REST levels in the brain following early life stress in rodents and found no significant changes (Uchida et al., 2010). Therefore, examining changes in REST protein within the amygdala using the recurrent animal model of depression would provide important insights into the role of REST in fear and anxiety-related behaviors, which are quite frequent in major depression.

5.6.3. How do Antidepressants and Recovery Influence Neurobiological Changes in the CORT Model of Recurrent Depression?

The experiments in this dissertation reveal novel findings of time-dependent bi-directional changes in hippocampal REST protein expression, as well as time-dependent increases in microglial activation through changes in cell morphology (Chapters 3 and 4). Although these data are supported by other research (Otsuki et al., 2010; Tynan et al., 2010a; Uchida et al., 2010), little is known how these neurobiological changes are rescued by antidepressant treatment or reversed by prolonged stress-free recovery. Interestingly, there is evidence to suggest that stress-induced atrophy of dendrites of pyramidal neurons in hippocampus is reversed upon stress cessation (Conrad et al., 1999). But the findings in this dissertation demonstrate that decreases in the number of DCX+ immature neurons in the dentate gyrus do not recover until the last recovery period (Chapter 3). More research is needed to determine whether a reversal of DCX+ cell loss is possible with longer recovery or whether these effects are dependent on the number of glucocorticoid re-exposures. To my knowledge, no research has been done on the effects of antidepressant medication on brain levels of REST protein. Taking into consideration that REST is highly involved in the

process of neurogenesis, which is known to be modulated by several classes of antidepressants (Banasr & Duman, 2007; Malberg et al., 2000; Santarelli et al., 2003), I hypothesize that a significant loss of REST as a result of multiple CORT treatments would be prevented in response to chronic antidepressant treatments.

Finally, several lines of evidence suggest that microglial function is modulated by several classes of antidepressant drugs, as well as the rapid antidepressant ketamine (Chang et al., 2009; Dubovický, Császár, Melicherčíková, Kuniaková, & Račková, 2014; Hashioka et al., 2007; Obuchowicz, Bielecka, Paul-Samojedny, 2014). However, more research is needed to investigate how these changes in microglial activation mediate depression-like behavior and hippocampal neurogenesis in a recurrent animal model of depression. Additionally, it would be critical to study whether microglial alterations recover following stress cessation or represent an irreversible damage that accumulates and contributes to depressive episode relapse.

5.7. Conclusions

The purpose of this dissertation was to develop an animal model of recurrent depression using repeated and cyclic CORT administration and to examine the neurobiological changes associated with depression recurrence in rats. The main and novel finding of this dissertation is that repeated and cyclic glucocorticoid exposure results in a behavioral aggravation of depression-like symptoms in rats. This result was replicated in two separate experiments with repeated and non-repeated behavioral testing; and moreover, aggravation was noted in both depression- and anhedonia-like behaviors. Examination of neurogenic markers in rats at different time points of the experiment revealed that the number and maturation rate of DCX+ immature neurons in the dentate gyrus were declined in rats with more CORT re-exposures. Furthermore, I found stress cycle-dependent bi-directional

changes in hippocampal REST protein expression, highlighting the importance of examining neurobiological changes across more than one exposure to stress. Changes in both neurogenesis and hippocampal REST levels were strongly correlated with increased depression-like behaviors. Finally, repeated and cyclic glucocorticoid treatment produced accumulative changes in microglial activation within the hippocampus, suggesting an additional mechanism mediating neurogenic and behavioral changes in an animal model of recurrent depression. In combination, the findings in this dissertation provide a novel way of studying recurrent depression and open new avenues of fruitful research investigating the neurobiological mechanisms governing depression relapse as well as new therapeutic approaches for preventing depressive episode recurrence.

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